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SECTION MEETINGS

CLEVELAND, O.	
University Hospitals	February 20, 1961
University Hospitals	March 20, 1961
DISTRICT OF COLUMBIA	
Georgetown University	March 30, 1961
ILLINOIS	
University of Illinois Med. School	March 14, 1961
IOWA	
State University of Iowa	March 29, 1961
MICHIGAN	
Lafayette Clinic, Detroit	March 16, 1961
MINNESOTA	
University of Minnesota	March 23, 1961
SOUTHERN	
Touro Infirmary, New Orleans	February 22, 1961
SOUTHERN CALIFORNIA	
University of Southern California	March 16, 1961
SOUTHWESTERN	
Southwestern Medical School	March 17-18, 1961

Identification of Poliovirus Isolates with Fluorescent Antibody. (26516)

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Isolation and identification of enteroviruses from clinical specimens by routine methods generally require 2 or more weeks. Fluorescent antibody procedures, which have been applied to rapid diagnosis of influenza by Liu (1) and to demonstration of several viruses in tissue culture(2,3), also appear to offer the possibility of more rapid identification of enteroviruses isolated from clinical material. This report presents data concerning use of fluorescent antibody for identification of poliovirus isolates. A preliminary report of some of these data has already been made(4).

Materials and methods. Hyperimmune monkey poliovirus typing sera prepared by Wenner *et al.*(5) were used for preparation of fluorescent antibodies. Trypsinized rhesus monkey kidney cells were employed for isolation of viruses. Buffered saline consisted of 0.14 M NaCl, 0.01 M $NaH_2PO_4 \cdot H_2O$, with the final pH adjusted to 7.0-7.2 by addition of 40% NaOH solution.

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Globulins were precipitated from monkey antisera by 50% saturation with $(NH_4)_2SO_4$ and labeled with either fluorescein isocyanate (6) or fluorescein isothiocyanate(7). The latter compound was added in powdered form to globulin solutions at pH 9 with stirring in an ice bath (personal communication, Riggs, 1958). Before use, conjugates were absorbed twice with monkey liver powder(8) and once with a suspension of monkey kidney cells.

For fluorescent antibody study, 85 stools previously tested by routine methods were selected from storage and given to 2 of the authors (MHH and GWA) who had no knowledge of the earlier results. The specimens were then processed entirely as unknowns. Ten to 20% stool suspensions were prepared, penicillin and streptomycin added to final concentrations of 4000 units/ml and 2000 μg/ml, respectively, and 0.1 or 0.2 ml portions inoculated into tubes of monkey kidney cells. As soon as a 1 to 2+ cytopathic effect (CPE) was observed, maintenance fluid was poured off and cells rinsed with 1 ml of 0.02% versene in buffered saline. One ml of fresh versene was added and tubes incubated about 15 minutes at 37°C. Cells were then removed from the glass by gentle shaking and sedimented by centrifuging at 2000 RPM for 5 minutes. Smears of sedimented cells were made on slides with a loop as in preparing bacterial smears. Slides were air dried and fixed in acetone at room temperature for 10 minutes. They were either stained immediately or stored at –20°C until stained. Control slides of uninoculated monkey kidney cells and known poliovirus infected cells were prepared at the same time slides were made on test specimens. All stools which produced a toxic reaction in monkey kidney cells were passed a second time. Staining in all these cases was done on second passage material. The majority of stools (21/27) producing no CPE in first passage were also passed a second time. In these instances, negative tubes were incubated 7 days, frozen, thawed and 0.1 ml passed to fresh tubes. Second passage tubes remaining negative were harvested at the end of 7 days for staining. Tubes on 6 stools that did not produce CPE in first passage were harvested and stained at the end of this passage. In many experiments, Leighton tube preparations of monkey kidney cells were also inoculated with stool suspensions. Coverslips from these tubes were removed at the 1 to 2+ CPE stage, air dried, and fixed in acetone as above. The direct method of fluorescent antibody staining was employed throughout. Conjugates were used undiluted or diluted 1:2 with buffered saline. All preparations were examined under ultraviolet illumination using a Corning 5970 or 5840 exciter filter. A Wratten 2B gelatin filter was used in the microscope ocular. Results of fluorescent antibody method were compared to those obtained previously with the routine neutralization method used for identification of polioviruses in this laboratory.

Results. Conjugates were checked in various ways for specificity of staining. In several cross staining experiments, smears or Leighton tube coverslip preparations of cells infected with poliovirus 1, 2 or 3 were stained with antipoliovirus 1, 2 and 3 conjugates. In each case, only the homologous system showed significant staining. Occasionally, slight cross staining was noted between poliovirus Types 1 and 3, but staining with homologous conjugate was always definitely brighter. Smears were also made from poliovirus infected tubes showing varying degrees of CPE. The brilliance of staining as well as the number of stained cells increased as CPE increased to about the 2+ stage. However, smears prepared from tubes showing 3+ or 4+ CPE did not stain well, possibly because of release and dispersion of viral antigen from cells. Similar observations were made with Leighton tube material. Lastly, inhibition tests were performed by the one-step method(9). When a mixture of equal parts of undiluted, unlabeled homologous monkey poliovirus antiserum and undiluted homologous conjugate was used to stain smears, staining was markedly inhibited. Significant inhibition of staining did not occur when unlabeled normal monkey serum was mixed with homologous conjugate. Results

TABLE I. Comparison of Fluorescent Antibody and Routine Procedure in Identification of Poliovirus Isolates.

Enterovirus type in stool		No. by routine procedure	No. by fluorescent antibody procedure
Polio 1		22	19
" 2		2	2
" 3		14	13
Non-polio*		47	47†
	Total	85	

* Eight Coxsackie B2, 3 Coxsackie B5, 1 Coxsackie A9, 1 ECHO 4, 5 ECHO 9, 29 no enteroviruses.

† Fluorescent antibody results negative with poliovirus conjugates indicating no poliovirus present.

of the comparison of fluorescent antibody and routine procedures are shown in Table I. Fluorescent antibody method correctly typed 34 of the 38 polioviruses present in the stools. In the remaining 4 cases, although the presence of virus was clearly recognized by CPE in tissue culture, there was no staining by the poliovirus conjugates. Where stools contained a Coxsackie or ECHO virus or no enterovirus, poliovirus fluorescent antibodies gave negative results. Thus, there were no false positive staining reactions in the 47 cases from which poliovirus was not isolated by routine methods.

Discussion. The reasons for the failure of fluorescent antibody staining to detect poliovirus in the 4 instances cited are not known. Two of these stools were reprocessed later, stained with fluoresecnt antibody and the viruses successfully typed as poliovirus, Type 1. However, on the basis of the data presented, fluorescent antibody is not a complete substitute for routine methods in poliovirus identification. Further studies are necessary to determine whether (a) better correlation can be obtained between fluorescent antibody and routine methods and (b) fluorescent antibody can be used as a screening procedure with routine methods being applied to those agents which fail to type with poliovirus conjugates. The absence of false positive reactions in the 47 stools from which poliovirus was not isolated and the correctness of typing in all 34 instances in which polioviruses were identified would suggest the possible application of fluorescent antibody as a screening tool.

Certain advantages of fluorescent antibody method should be pointed out. First, laboratory identification of polioviruses in the stool could be made more rapidly with fluorescent antibody. One-half (17/34) the polioviruses were identified in first passage in tissue culture, generally within 72 hours after inoculation. In the remainder of the positive cases, second passage in tissue culture was necessary. In all but one instance this was due to toxicity of the stool suspension for monkey kidney cells. Even where more than one passage was necessary, fluorescent antibody was more rapid than routine procedure because of the titrations and neutralizations involved in the latter method.

Second, fluorescent antibody method was comparatively simple to carry out. Less material, particularly monkey kidney tissue cultures ,and fewer manipulations (i.e., pipetting, reading of tissue culture tubes) were involved in fluorescent antibody method. Such advantages might be offset to a certain extent by the rather expensive equipment necessary for fluorscence microscopy and by the time and experience required to initiate fluorescent antibody procedures. Over a long period of time, however, fluorescent antibody methods should prove simpler and more economical.

Where both Leighton tube preparations and smears were stained, both were satisfactory for identification of the isolate. However, recognition of infected cells after staining appeared somewhat easier with Leighton tube material. Characteristic fluorescent granules were frequently seen in the cytoplasm of infected cells stained *in situ* on the coverslips. In smear preparations, fluorescence tended to be seen as larger cytoplasmic masses, and all cells appeared rounded as a result of versene treatment.

Summary. A specific and relatively rapid direct fluorescent antibody staining method for laboratory identification of polioviruses isolated from stool samples is reported. Eighty-five stool specimens were processed as unknowns in a comparison between the routine method employing neutralization tests

and the fluorescent antibody method. Thirty-eight were found to contain poliovirus Types 1, 2 or 3 by the regular procedure, and 34 of these were correctly identified by the fluorescent antibody method. The other 4 of the 38 were negative by fluorescent antibody. The 47 specimens found not to contain poliovirus by the routine method were negative by fluorescent antibody. Eighteen of these contained other enteroviruses. Thus, although the fluorescent antibody procedure was found slightly less sensitive than the routine procedure, it was entirely specific in this study.

1. Liu, C., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 883.

2. Coons, A. H., *Internat. Rev. Cytol.*, 1956, v5, 1.

3. Buckley, S. M., *Arch. f. Virusforsch.*, 1956, v5, 388.

4. Kalter, S. S., Hatch, M. H., Ajello, G. W., *Bact. Proc.*, 1959, 89.

5. Wenner, H. A., Miller, C. A., Kamitsuka, P. A., Wilson, J. C., *Am. J. Hyg.*, 1954, v59, 221.

6. Coons, A. H., Kaplan, M. H., *J. Exp. Med.*, 1950, v91, 1.

7. Riggs, J. L., Seiwald, R. J., Burckhalter, J. H., Downs, C. M., Metcalf, T. G., *Am. J. Path.*, 1958, v34, 1081.

8. Coons, A. H., Leduc, E. H., Connolly, J. M., *J. Exp. Med.*, 1955, v102, 49.

9. Goldman, M., *ibid.*, 1957, v105, 557.

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Evidence for an Hypothalamic Focus of Inhibition of Gonadotropin by Androgen in the Male.* (26517)

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Control of pituitary gonadotropic secretion is generally acknowledged to be partially a function of the level of circulating gonadal hormones. The view has recently become widespread that in the female this "feedback" influence of sex hormones is an indirect one, operating through the hypothalamus, wherein lie receptor structures which respond to the sex steroids by modifying adenohypophysial activity(1,2,3). This report presents evidence that also in the male an area within the hypothalamus responds to the gonadotropin-inhibiting influence of the sex-specific gonadal hormone, testosterone.

Methods. Testosterone propionate was implanted into the brains or hypophyses of 11 mongrel male dogs. The crystalline steroid was heated in a glass tube, and immediately on reaching the melting point some of the hormone was drawn by capillary action into the end of 22 gauge stainless steel tubing. After cooling, the steel tube was cleaned on the outside with solvents, and a minute amount of solid testosterone (of the order of 0.5 mg) retained at the tip. With the aid of the dog stereotaxic instrument(4) tubes so prepared were implanted through the calvaria, each tip being located in the brain or hypophysis as described in the Table.

Semen ejaculates were collected and evaluated as described previously(5). Sperm count and motility were measured on 2 to 4 occasions (6-20 days) before implantation and at intervals after implantation of testosterone. The dogs were autopsied 24-33 days postoperatively. Testes and prostates were removed, weighed, fixed in SUSA solution, sectioned, and stained with hematoxylin and eosin. Brains were perfused and fixed in 10% formalin and sectioned by the freezing method for location of testosterone implantation sites. After removal of the tubes, testosterone was generally still clearly visible at their tips.

Results. Only dogs with pre-implantation ejaculates containing over 100 million highly motile spermatozoa were used in this study. In one group (Table I, Group I) implantation of testosterone into the ventral hypothalamus was followed by failure of ejaculation. In the case of dogs A and G, the first postoperative

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TABLE I. Effects of Implanting Testosterone Propionate into Brain or Pituitary Gland of the Male Dog.

Group	Dog	Location of implant	Testicular wt (mg/kg body wt)	Testicular histology	Prostatic histology
I	A	Immediately above post median eminence	1,130	Moderately severe atrophy*	Atrophy
	C	Posterior tuber	711	Complete atrophy†	”
	G	Mid median eminence	653	*Idem*	”
	K	Post median eminence	617	”	”
II	F	Pituitary	1,395	Normal	Normal
	H	”	1,550	”	”
	I	”	1,890	”	”
	L	” ‡	1,080	Some tubular disorganization	Slight degeneration
III	D	Thalamus	1,480	Normal	Normal
	E	”	1,280	”	”
	J	Ventromedial area of hypothalamus	1,320	”	”

* Somewhat less atrophied than the condition in hypophysectomy.
† Similar to condition in hypophysectomy.
‡ Location of implant not verified *post mortem*.

attempt to collect semen (one week after implantation), and all other attempts, were unsuccessful. In dogs C and K there was a gradual decrease to zero in sperm count and motility. Animals with pituitary implants (Group II) showed in 2 cases (dogs F and I) little or no deterioration of ejaculates following implantation, while in the 2 others there was a sharp drop in semen quality with eventual failure of ejaculation in dog H. In dogs of Group III, seminal ejaculates remained normal and essentially unchanged. The location of the testosterone implant in each of the animals is shown diagrammatically in Fig. 1.

A striking difference between the effects of testosterone implantation in the pituitary and in the posterior median eminence area becomes obvious from consideration of the parameters of testicular function in Table I. Testicular weights in the 4 dogs of Group I averaged 0.773 g/kg body weight; in Group II, 1.48 g/kg, and in Group III, 1.36 g/kg. Histologically, all animals of Group I showed marked gonadal atrophy (Fig. 2). In the testes of dogs C, G and K, spermatozoa were absent, spermatids absent or rare, and many seminiferous tubules were lined with Sertoli cells and spermatogonia alone. The lumina of the tubules were closed with amorphous material. In dog A a few scattered spermatozoa remained, but many tubules were disorganized and closed. It is of interest that in this animal the testosterone implant was not quite inside the posterior median eminence, but a little above it (Fig. 1). In all 4 dogs

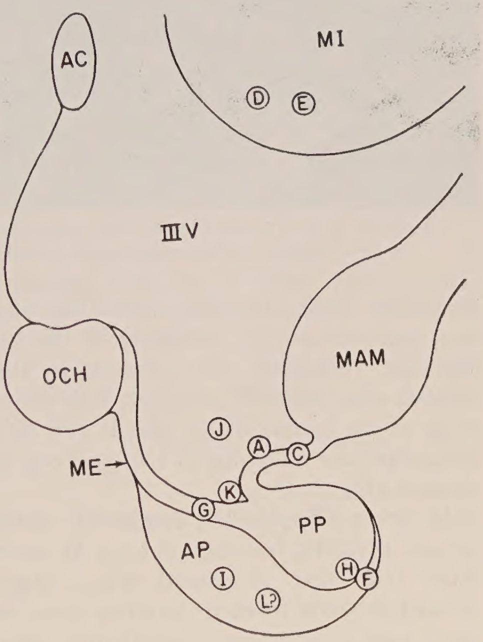

FIG. 1. Location of testosterone implants in dog brain and pituitary. Abbreviations: AC, anterior commissure; AP, anterior pituitary or pars distalis; MAM, mammillary body; ME, median eminence; MI, massa intermedia; OCH, optic chiasm; PP, posterior pituitary or infundibular process; III V, 3rd ventricle.

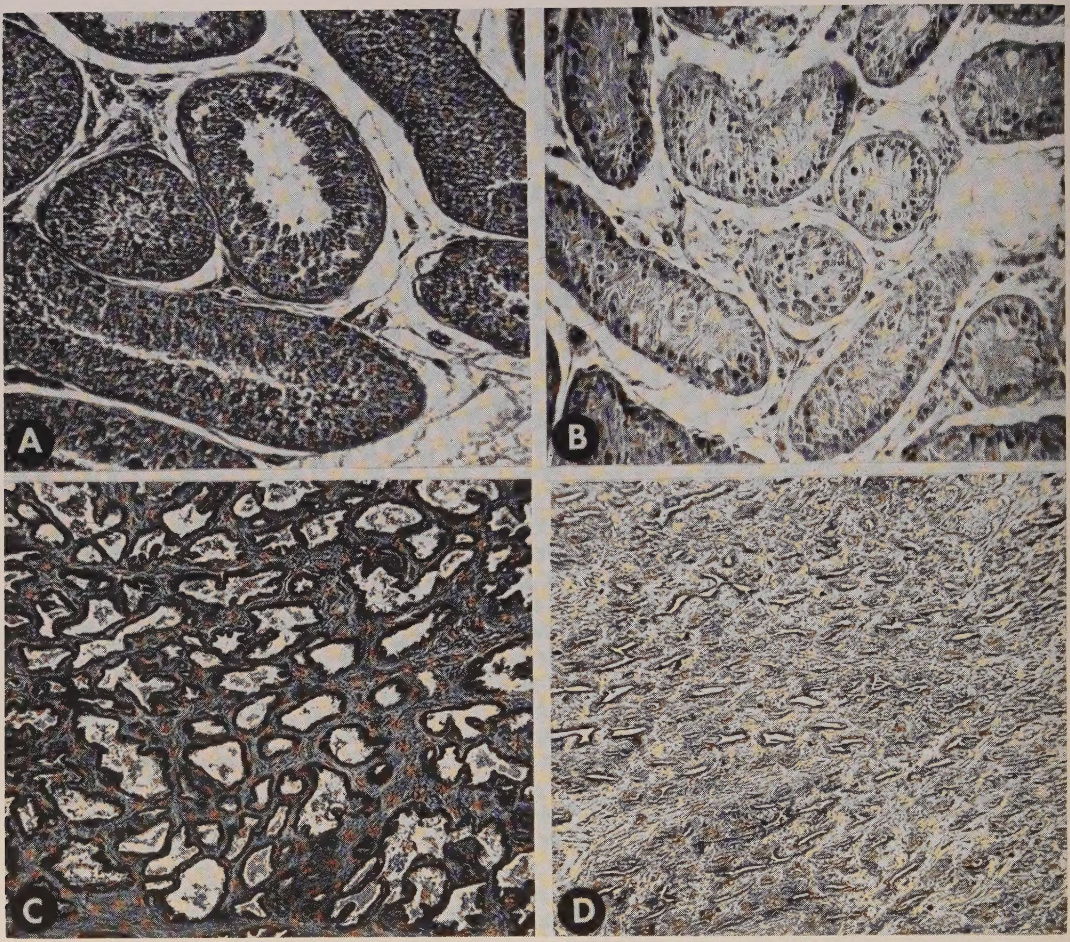

FIG. 2. A, C—Testis (×150) and prostate (×40) of Dog F (testosterone in pituitary gland). B, D—Testis (×150) and prostate (×40) of Dog G (testosterone in mid median eminence).

of Group I the testicular interstitial tissue was characterized by involution of the Leydig cells. Similarly, the prostates in these animals were markedly atrophic, with obliteration of the lumina of the glands and disorganization and flattening of the secretory epithelium (Fig. 2, D).

In Group II (pituitary implants), testicular and prostatic histology did not in general differ from that of normal dogs. Dog L showed the only marked deviation from normal: some disorganized seminiferous tubules and small prostatic gland lumina with, however, a generally columnar epithelium. Both testes and prostate of Dog L had a decidedly more healthy appearance than those of Dog A, the animal with the least degree of atrophy in Group I. Although the location of the implant in Dog L was not confirmed *post mortem,* its position in radiographs appeared to be definitely hypophysial.

Discussion. In male dogs(6,5) as well as in males and females of other species(7-10) destruction of the posterior median eminence region has been shown to lead to gonadal atrophy. This condition is apparently due to inhibition of production and secretion of FSH and ICSH(11). But although the hypothalamus appears to be essential for gonadal maintenance, its role in the "negative feedback" mechanism whereby gonadal hormones influence gonadotropin secretion has not yet been clearly established. Evidence that estrogen acts directly on hypothalamic structures to reduce gonadotropin secretion comes from experiments in female animals, involving transplantation of ovarian tissue to the hypothalamus(1) and implantation of solid estrogen therein(2,3).

Hohlweg and Daume(12) injected estrogen in fluid medium into the diencephalon of male birds and rats, with resulting gonadal atrophy. Their work, however, did not demonstrate the localization of the "feedback center," for the estrogen might well have diffused to the pituitary and acted there. The present study indicates that the fixed local implantation of minute amounts of solid androgen into the posterior median eminence area results in aspermia and profound atrophy of testes and prostate similar to that found in hypophysectomized dogs or those with lesions in the posterior median eminence. This effect was not seen with similar implants in the pituitary gland or some other areas of the brain.

It seems clear that the gonadal and prostatic atrophy observed here must be ascribed to the presence of testosterone in the median eminence. The possibility is remote that the results were due to mechanical destruction of the tissue by introduction of steel tubes, for the following reasons: (1) In the case of dogs C and G, the tubes were located just outside the critical area, destruction of which has previously been shown to lead to gonadal atrophy. 2) The pituitary implantations must have entailed at least as much damage to the median eminence as the implantations aimed directly at this structure. The tubes in dogs H and F (group II) passed through exactly the same areas of brain as those of dog C (group I).

It is concluded that this study provides positive evidence for the view that in the male dog gonadotropin secretion is held in check by the action of androgen on the posterior median eminence, and that the latter site is a critical area involved in the "negative feedback" of androgen on pituitary function.

Summary. The chronic implantation of minute amounts of solid testosterone propionate into the posterior median eminence-posterior tuberal region of the hypothalamus in 4 dogs has led to aspermia and profound testicular and prostatic atrophy. Although decreases in quality of semen were noted in 2 of 4 other dogs with similar implants in the pituitary, none of these 4 dogs showed gonadal or prostatic atrophy. No decrease in semen quality nor gonadal or prostatic atrophy was noted in either of 2 dogs with thalamic implants or one with an implant in the ventromedial hypothalamus.

The authors would like to thank Enid Arozena, Cora Rucker and Arlene Koithan for skilled technical assistance.

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1. Flerko, B., Szentagothai, J., *Acta Endocrin.*, 1957, v26, 121.

2. Lisk, R. D., Personal communication, 1960.

3. Davidson, J. M., Sawyer, C. H., *Acta Endocrin.*, 1961, In press.

4. Hume, D. M., Ganong, W. F., *EEG clin. Neurophysiol.*, 1956, v8, 136.

5. Davidson, J. M., Ganong, W. F., *Endocrinology*, 1960, v66, 480.

6. Ganong, W. F., Frederickson, D. S., Hume, D. M., *ibid*, 1955, v57, 355.

7. Dey, F. L., *ibid.*, 1943, v33, 75.

8. McCann, S. M., *Am. J. Physiol.*, 1953, v175, 13.

9. Sawyer, C. H., Robison, B., *J. clin. Endocrinol. & Metab.*, 1956, v16, 914.

10. Sawyer, C. H., *J. Exp. Zool.*, 1959, v142, 227.

11. Davidson, J. M., Contopoulos, A. N., Ganong, W. F., *Endocrinology*, 1960, v66, 735.

12. Hohlweg, W., Daume, E., *Endokrinologie*, 1959, v38, 48.

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Observations on Cytotoxic Effect of Antihistiocyte Serum.* (26518)

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Guinea pig anti-rabbit histiocyte serum exerts a cytotoxic effect on living histiocytes in the presence of high concentrations of complement. Although cytotoxic action of antibody, especially on tumor cells, has been reported(1-7), the role of whole complement or complement components in the cytotoxic reaction has been neglected until recently(8). This paper describes preliminary observations on some of the characteristics of guinea pig anti-rabbit histiocyte antibodies and their cytotoxicity for live histiocytes.

The cell designated as a histiocyte in this paper is a large mononuclear cell elicited by oil in the peritoneal cavity of the rabbit. It has morphologic and tinctorial characteristics very similar to the blood monocyte and is synonomous with the wandering macrophage.

Materials and methods. Antibody production. Whole histiocytes harvested in the manner given below were mixed into a modified Freund's adjuvant (no BCG added). Each guinea pig received 3 doses of one ml antigen subcutaneously at weekly intervals. One week later, 0.25 ml saline-suspended histiocytes were injected into each foot pad. Sensitization was followed by measuring precipitating antibody against soluble antigens extracted in Grabar's solution conservatrice(12) from histiocytes which were ground in liquid nitrogen. From 10 to 30 mg of antigen (as whole cell nitrogen) was required per animal for production of precipitating antibody against these soluble antigens.

Histiocyte production. Histiocytes were harvested from the peritoneal cavity of New Zealand white rabbits (3 to 5 lb) 5 days after injection of 50 ml of Klearol. Cells were washed from the cavity with Grabar's solution conservatrice (SC)(12). Oil was separated from cells by separatory funnel. Cells were sedimented at 250 g for 10 minutes and washed 3 times in SC. Packed cells were used for the immunizing antigen. About 10 to 20% of the cells were non-viable at time of harvest.

Complement (C′). Commercial,‡ dehydrated guinea pig complement was reconstituted according to package directions.

Dye. 1% trypan blue in 0.85% saline, filtered and diluted 1:5 in saline, was used in cytotoxic tests.

Cytotoxic test. To a 4-inch screw cap test tube was added: 1.0 ml antiserum diluted 1:10 or more, 1.0 ml cell suspension (1×10^6/ml in 0.85% NaCl and 5% bovine serum albumin), 0.5 ml 0.0012 M Ca^{++} solution in 0.85% NaCl and 0.5 ml 0.004 M Mg^{++} solution in 0.85% NaCl. The mixture was brought to 37°C in a water bath. One ml of complement, diluted 1:5 and brought to 37°C, was added. The test mixture was incubated 60 minutes at 37°C; tubes were frequently hand-mixed. The mixture was centrifuged for 2 minutes, supernatant discarded, and cells resuspended in 0.5 ml diluted trypan blue. The cell-dye suspension was held at room temperature (25°C) for 3 to 5 minutes. A drop of the mixture was placed on 3 × 5 microscope slide covered with 22 × 40 mm coverslip, and examined by high power objective. The number of cells with blue-stained nuclei in a total of 200 cells was recorded. The percent dead cells was corrected on the basis of 100% live cells in the control suspension.

Kinetic tests. For the kinetic studies multiples of the 4.0 ml test dose were placed in a flask, samples withdrawn periodically and treated as described above. Time was measured from the moment of addition of complement. Unless otherwise noted, all reagents were brought to 37°C before adding complement.

Experimental results. The cytotoxic activity of the antihistiocyte serum was heat stable

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* Aided by funds available on grant from Nat. Inst. Health.

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TABLE I. Cytotoxic Effect of Antihistiocyte Serum in Presence of Varying Amounts of Complement. (Activity recorded as % cells killed.)

	ml complement			
ml serum	.05	.1	.2	.4
.1	72	80	82	92
.05	32	71	57	78
.025	28	40	43	69
.0125	16	26	20	41

(56°C, 1 hour) and could be absorbed from the serum by whole live histiocytes without qualitatively affecting the serum's gel diffusion pattern with soluble antigens. A reciprocal relationship was observed between amount of antibody and amount of complement which gave maximal killing effect. (*See* Table I).

No extra calcium or magnesium ions were added to the system in this experiment, and SC solution was employed instead of normal saline.

In excess complement, maximal rate and extent of killing was dependent upon amount of antibody present in the system. Maximal cytotoxicity of 0.05 ml of antiserum pool used in these experiments was reached 50 minutes after addition of complement.

Combined effect of calcium and magnesium ions on cytotoxic reaction. Two flasks were set up, one with 10 test doses for the control, (I) the other with 30 test doses (II). No additional Ca^{++} or Mg^{++} was added to flask II. Disodium ethylenediamine tetraacetate (EDTA-50μM) was added, which was enough to bind the estimated Ca^{++} and Mg^{++} in antiserum and in complement solutions. Four ml portions were removed from each flask over a period of 50 minutes after complement was added. At 60 minutes the remaining contents of flask II were divided (flask IIa and IIb), and Ca^{++} and Mg^{++} sufficient to tie up all the EDTA, with excess for maximal free ion concentration, was added to flask IIa. Additional samples were removed and counted from both flask IIa and flask IIb over a period of 50 minutes.

Fig. 1 indicates that the cytotoxic reaction was dependent on one or more of the divalent cations. Increased killing in the EDTA control after 80 minutes was possibly due to pH changes, as the system was not buffered.

When no Ca^{++} or Mg^{++} was added, minimal killing effect was observed over a period of 70 minutes. If Mg^{++} alone was added, the reaction was maximal without addition of Ca^{++}. There was an optimal concentration of Ca^{++} in the minimal system due to the calcium concentration in guinea pig serum present, whereas concentration of Mg^{++} was sub-optimal. The results indicate a requirement for Mg^{++}, but do not indicate whether or not Ca^{++} is required.

The reaction of complement in the cytotoxic system was compared to the reaction in the sheep red cell-rabbit hemolysin system. Sensitized histiocytes were subjected to complement, Ca^{++} and Mg^{++} at 0°C for one hour in an attempt to form HAC$'_{142}$ cells analogous to EAC$'_{142}$ cells of Levine *et al.*(9,10). These cells are designated as C$'_{0_0}$ cells and the supernatant removed from these cells as C$'_{0_0}$ super. The antiserum did not kill the cells in the presence of complement at 0°C. Fig. 2 presents data comparing the rate of cytotoxic reaction when the following supernatants at 37° were added to the C$'_{0_0}$ cells: C$'_{0_0}$ super (Curve 1); fresh complement with no additional Ca^{++} or Mg^{++} (Curve 2); heated complement (15 minutes 56°C) (Curve 3) and fresh complement in the presence of 0.02M EDTA (Curve 4). There was no difference between curve 4 and its control with EDTA but without complement. Each mixture was incubated at 37°C and sampled as described above. The results indicate that HAC$'_{142}$ cells were not formed at 0°C because neither the heated complement nor the EDTA-treated complement, as sources of C$'$3, mediated the cytotoxic reaction. There is evidence that pretreatment of the cells shortened the time necessary for the reaction to reach a maximum. C$'_{0_0}$ cells formed in the presence of 0.001 M Ca^{++} and 0.003 M Mg^{++} according to Becker's method(11), gave the same rate and extent of reaction as C$'_{0_0}$ cells formed in presence of the amounts used routinely.

To determine if any complement activity was removed from the supernatant by sensitized cells at 0°C, C$'_{0_0}$ super (37°C) was added to fresh sensitized cells, and to C$'_{0_0}$

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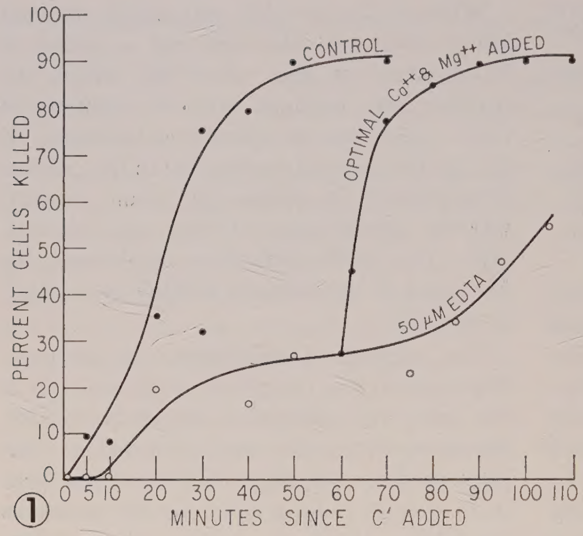

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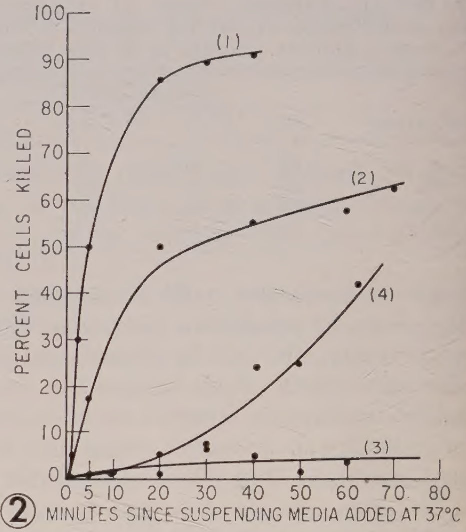

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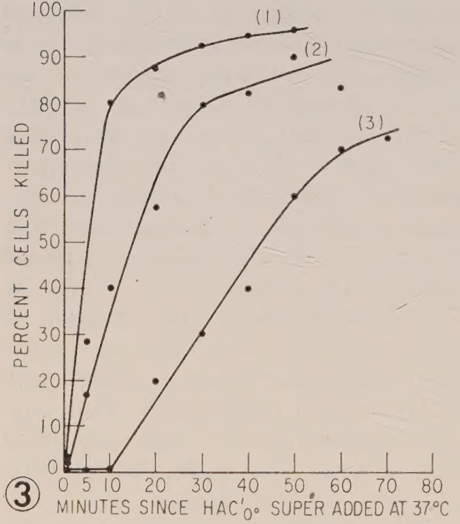

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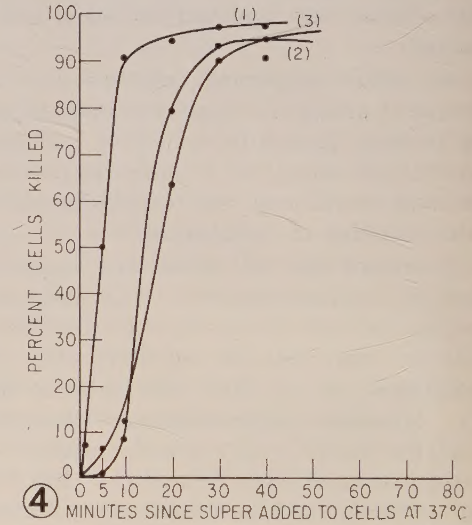

FIG. 1. Effect of divalent ions (Ca^{++} and Mg^{++}) on rate of cytotoxic reaction of antihistiocyte serum.

FIG. 2. Effect of addition of various suspending media to HAC'_{O° cells at 37°C on rate of cytotoxic reaction. (Curve 1) C'_{O° super; (Curve 2) fresh complement with no additional Ca^{++} or Mg^{++}; (Curve 3) heated complement; (Curve 4) fresh complement in presence of 0.02 M EDTA.

FIG. 3. Rate of cytotoxic reaction of HAC'_{O° super on: (Curve 1) HAC'_{O° cells, (Curve 2) fresh, sensitized cells, (Curve 3) unsensitized cells at 37°C.

FIG. 4. Effect of complement previously treated with unsensitized histiocytes on: (Curve 1) previously sensitized cells, (Curve 2) unsensitized cells after antiserum is added to the altered super. Curve 3 shows cytotoxic reaction of supernatant fluid taken from sensitized cells on fresh cells (test for unfixed antibody).

cells (37°C) as control. Addition of C'_{O° super to sensitized cells which had not had previous contact with complement resulted in cytotoxicity at a slower rate, and to a lower maximum (Fig. 3, curve 2) than occurred in the control reaction (Fig. 3, curve 1). The reaction of C'_{O° super with unsensitized cells (Fig. 3, curve 3) suggested residual antibody activity in the C'_{00} super. Evidence for residual antibody is seen in Fig. 4, curve 2, which shows the cytotoxic reaction of supernate removed from sensitized cells (to which complement had not been added) and in turn added to fresh unsensitized cells in presence of complement.

It was possible that histiocytes alone affected complement and resulted in production of a toxic material. Histiocytes were treated with complement at 0°C in the absence of antibody. The supernatant of these cells, designated "altered" C'_{00} super, was tested for cytotoxic activity by adding it to fresh unsensitized cells at 37°C. The results shown in Fig. 4, curve 1, show that preincubation of complement with cells alone had no effect on the rate of the reaction. Fig. 4, curve 3, shows the effect of "altered" C'_{00} super (mixed with antiserum at 37°C) added to fresh cells as compared to the reaction of "altered" C'_{00} super added to previously sensitized cells (Fig. 4, curve 1). The lag evident in curve 3 suggests that sensitization of the cells is a rate-limiting reaction under the conditions of the experiment.

Discussion. The results of these preliminary experiments suggest that the reaction of guinea pig complement in the histiocyte immune system differs in certain characteristics from its reaction in the red cell immune system. The inability to form $C'142$ cells at 0°C recalls the human complement red cell system. The large amount of complement necessary in the histiocyte system is not explained by anticomplementary effects of the reaction constituents. Tests using reagents of complement(13) show that R1, R2, R3 (the latter prepared both by formalin(14) and Zymosan), E, M & H do not replace fresh guinea pig serum, supporting the view that complement is the constituent which is active in the cytotoxic system.

Summary. Preliminary observations of the cytotoxic action of guinea pig antirabbit histiocyte serum mediated by large amounts of guinea pig complement have been presented. The data suggest that guinea pig complement does not act in the same manner in this system as it does in the sheep-red cell system with respect to the amount required and to its action at 0°C.

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1. Bjorklund, B., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 1.

2. Lumsden, T., *Am. J. Cancer,* 1937, v31, 430.

3. Mountain, I. M., *J. Immunol.,* 1955, v75, 478.

4. Plescia, O. J., *Cancer Res.,* 1956, v16, 743.

5. Ross, J. D., *Ann. N. Y. Acad. Sci.,* 1957, v69, 795.

6. Schrek, R., Preston, F. M., *Cancer Res.,* 1957, v17, 102.

7. Wissler, R. W., Flax, M. H., *Ann. N. Y. Acad Sci.,* 1957, v69, 773.

8. Ross, A., Lepow, I. H., *J. Exp. Med.,* 1960, v112, 1085.

9. Levine, L., Mayer, M. M., Rapp, H. J., *J. Immunol.,* 1954, v73, 435.

10. Mayer, M. M., Levine, L., Rapp, H. J., Marucci, A. A., *ibid.,* 1954, v73, 443.

11. Becker, E. L., *ibid.,* 1956, v77, 462.

12. Grabar, P., Seligmann, M., Bernard, J., *Ann. Inst. Pasteur* (Paris), 1955, v88, 548.

13. Kabat, E. A., Mayer, M. M., *Experimental Immunochemistry,* 1948, C. C. Thomas, Springfield, Ill., p. 122.

14. Rodriquez, E., Osler, A. G., *J. Immunol.,* 1960, v85, 347.

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Studies on the Pathogenesis of Experimental Immune Thyroiditis.* (26519)

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Immunization with homologous or autologous thyroglobulin is followed in a high proportion of experimental animals by a chronic inflammatory reaction in the thyroid gland, which bears some resemblance to human chronic lymphocytic thyroiditis(1,2). It has been established that thyroglobulin is an antigen capable of producing this experimental autoimmune disease. Whereas much investigation has been devoted to the question of the antigenicity of thyroglobulin, the serology of antithyroglobulin antibody and the course of this experimental immune thyroiditis, the pathogenetic mechanism has not been elucidated(3,4). Circulating antibody directed against thyroglobulin has been considered to be an important pathogenic factor; however, the correlation between circulating antibodies and incidence or degree of thyroiditis is at best vague. In addition, it has been impossible to transfer the disease passively with massive doses of high titer antithyroglobulin serum.

The experiments described here were designed to study the problem of whether incidence of experimental immune thyroiditis in the guinea pigs bears a closer relationship to formation of circulating antibodies or to delayed type of hypersensitivity against thyroglobulin. Use was made of the fact that when minimal amounts of the immunizing antigen are heavily conjugated with a haptene (picryl chloride), serum antibody production is decreased without interfering with the ability of the antigen to induce delayed type of hypersensitivity.

Materials and methods. Thyroglobulin was prepared according to the method of Derrien(5) as modified by Roitt. Thyroid glands of 80 guinea pigs were frozen immediately after removal. The tissue was cut into very thin slices while maintained in a frozen state. One part of thyroid tissue was placed in 3 parts by weight of physiological saline and kept overnight at 4°C. The saline extract was subsequently filtered through surgical gauze and centrifuged at 8000 g for 10 minutes at room temperature. One hundred ml of the clear supernatant was brought to 42% ammonium sulfate saturation by the dropwise addition of 72.5 ml of saturated ammonium sulfate solution at room temperature. The 42% saturated ammonium sulfate extract was stirred for 30 minutes and the precipitate was then centrifuged at 6000 g for 10 minutes at room temperature. The supernatant was discarded and the precipitate redissolved in 50 ml of a 45% saturated ammonium sulfate solution. Subsequently, the ammonium sulfate concentration was slowly decreased by dropwise addition of 10.75 ml of distilled water to a final ammonium sulfate saturation of 37%. The suspension was continuously stirred for 2 hours, then centrifuged at 6000 g for 10 minutes. The thyroglobulin left in the supernatant was precipitated by dropwise addition of 4.1 ml of a solution of saturated ammonium sulfate (final saturation = 42%). The precipitated thyroglobulin was finally centrifuged at 6000 g at room temperature for 10 mintues and the supernatant subsequently discarded. The precipitated thyroglobulin was dissolved in saline and dialyzed against saline. Nitrogen content was determined by the micro Kjeldahl method (6.8 mg N/ml).

Preparation of picrylated thyroglobulin. 0.1 g of crystallized picryl chloride was dissolved in acetone and mixed with 1.9 g of cellulose (Hyflo-Super-cel, Johns Manville, COMPOC Calif.). The acetone was evaporated and a concentration of 5% picryl chloride adsorbed on cellulose remained.

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Twenty-five ml of a 4 mg/ml solution of guinea pig thyroglobulin was brought to pH 8.5 by dropwise addition of a solution of 10% sodium carbonate. This solution was mixed with 300 mg of the picryl chloride cellulose powder (equivalent to 15 mg of picryl chloride). This procedure results in slow picrylation of thyroglobulin. With increasing picrylation of the protein, the pH of the solution falls. The pH was continuously adjusted to 8.5 by dropwise addition of 10% sodium carbonate. Picrylation was continued for 6 hours with constant stirring of the solution. At the end of this period, the solution was centrifuged and the supernatant withdrawn. The powder was suspended in an equal amount of distilled water and recentrifuged. The supernatant was mixed with the previous one and dialyzed against distilled water for 2 days(6).

Immunization of guinea pigs. Two groups of guinea pigs were immunized with picrylated thyroglobulin, emulsified in an equal amount of complete Freund's adjuvant. One group of guinea pigs were immunized with a total of 500 μg of thyroglobulin, the other with 50 μg administered in each case in 2 series of injections separated by a 7-day interval. Initial injections were in the 2 front foot pads, the second injections in the rear foot pads. The material was administered in a 0.1 ml volume in each foot pad. Seven to 10 days after the second injection, the animals were skin tested by intradermal injection of 50 μg thyroglobulin in 0.1 ml of normal saline. The skin reaction was read and graded at the end of 24 hours as described elsewhere(6). The presence of circulating antithyroglobulin antibody was determined by the passive hemagglutination technic(7).§ The animals were sacrificed after the skin tests were read and blood was removed for antibody determination. Complete autopsies were performed and sections of thyroid gland, liver, spleen, adrenal, lung and kidney were taken. Hematoxylin and eosin stained sections were made of both lobes of the thyroid gland and sections of the other organs were examined in most animals.

Other groups of guinea pigs were immunized in a parallel series of experiments with tissue homogenates of the adrenal, liver and kidney emulsified in Freund's adjuvant as described above. Untreated animals maintained under the same conditions with identical housing and diet served as an additional control group.

Examination and evaluation of thyroid gland. Both lobes of the thyroid gland were examined in each animal and compared with control animal groups. The lesions were graded according to the following criteria: (+) Focal collections of inflammatory cells with some swelling of scattered follicular epithelial cells. Occasional desquamation of epithelial cells and macrophages within the follicles. No gross alteration of colloid. (++) Confluent areas of inflammation consisting principally of lymphocytes and plasma cells, but without extensive derangement of the normal follicular architecture. Moderate to marked colloid depletion associated with a varying degree of alteration was noted. (+++) Gross disruption of the normal follicular pattern with generalized involvement of both lobes. Areas of granulomatous inflammation in which no recognizable normal structures could be identified.

Results. At the end of the experimental period the animals immunized with thyroglobulin were in good condition and appeared to have gained weight at approximately the same rate as the control groups.

In the group of animals immunized with a course of 50 μg of picrylated thyroglobulin 30 of 43 or 69% developed delayed type of hypersensitivity to thyroglobulin. Of these 8 had strong reactions while 22 had weaker though distinctly positive reactions. Two of the 43 or 4.7% had circulating antithyroglobulin antibody. Twenty-five of the 43 animals developed thyroiditis. In the animals immunized with 500 μg picrylated thyroglobulin, incidence of delayed type of sensitivity was 74% (47 of 67 animals). Ten of these displayed a strong skin reaction to thyroglobulin while 37 had somewhat weaker reactions. Twenty-one of 67 animals or 31% had demonstrable circulating antibody, 38 of

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§ The authors wish to acknowledge thanks to Dr. Noel R. Rose, Univ. of Buffalo School of Medicine, for checking the titers of many sera in his laboratory.

TABLE I. Incidence of Thyroiditis and Immune Response to Thyroglobulin in Animals Immunized with Picrylated Thyroglobulin.

		% of animals					
				Thyroiditis			
Picrylated thyroglob., µg	No. of animals	Circulating antibody	Delayed hypersensitivity	+	++	+++	Total
50	43	4.7	69.7	44	9.3	4.7	58
500	67	31.3	74	35.8	16.5	4.6	56.8

the 67 developed thyroiditis. These data are summarized in Table I. In addition, incidence of circulating antibody was approximately the same in the animals with thyroiditis (9 of 31) and those without any lesion (9 of 29). The percentage of animals with titers above 512 was slightly higher in the former group (Table II). Of a total of 61 animals with thyroiditis in both groups, 50 or 82%, had a positive delayed skin test. Sixteen of 61 animals or 26% had strongly positive reactions in this group. In a group of 45 animals without thyroiditis 62% or 28 of 45 had positive skin reactions. Only one of these animals had a strongly positive skin test (Table III).

No lesions were found in any other organ of the animals immunized with picrylthyroglobulin, with the exception of the liver and lung where small infarcts were occasionally noted. In one instance, a small foreign body granuloma in the lung was seen. This was interperted as a reaction to one of the constituents of Freund's adjuvant. Fig. 1 shows the histologic pattern of the thyroid gland of a typical control animal. Inflammatory cells were never observed. The control animals included groups immunized with Freund's adjuvant alone, and others immunized with organ extracts of liver, adrenal and kidney in Freund's adjuvant. Figures 2 and 3 demonstrate the typical lesion graded as ++ in a guinea pig which received a total immunizing dose of 500 µg picrylthyroglobulin. There is marked atrophy of the parenchyma with loss of colloid in addition to a generalized infiltration by mononuclear cells, principally plasma cells, lymphocytes and a few macrophages.

TABLE II. Circulating Antibody in Guinea Pigs Immunized with a Total Dose of 500 µg.

	No. of animals	Circulating antibody titer		
		16-512	512	Total
		%	%	%
Thyroiditis	31	12.9 (4)*	16.1 (5)	29 (9)
No thyroiditis	29	24.1 (7)	6.9 (2)	31 (9)

* No. of animals in parentheses.

In the group of 35 animals immunized with liver, adrenal, and kidney extracts in Freund's adjuvant, no lesion which could be classified as + was noted in the thyroid gland.

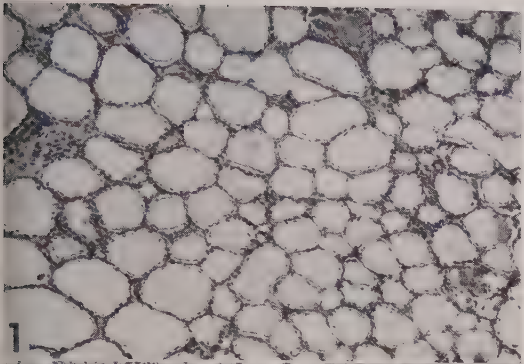
TABLE III. Comparison of Severity of Thyroiditis with Intensity of Delayed Skin Reaction to Thyroglobulin.

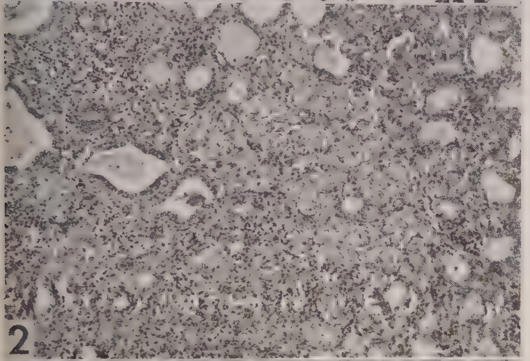
	Degree of thyroiditis	% of animals with positive delayed skin test to thyroglobulin	
61 animals with thyroiditis	+	52.4	39.4 weak*
			13 strong†
	++	21.4	11.6 weak
			9.8 strong
	+++	8.2	5.0 weak
			3.2 strong
	Total	82	56 weak
			26 strong
45 animals without thyroiditis		62	58 weak
			4 strong

* Weak reaction characterized by moderate induration and erythema.

† Strong reaction with marked induration usually associated with hemorrhage.

Discussion. The results of this series of experiments clearly indicate that there exists a close relationship between incidence of delayed type of hypersensitivity to thyroglobulin and incidence of immune thyroiditis in this experimental model. No such correlation could be found with respect to circulating antibodies. These conclusions are based in part on the special exprimental conditions, utilizing the fact that in the course of immunization with minimal doses of antigen there is a phase in the immune response where little or no circulating antibody is demonstrable.





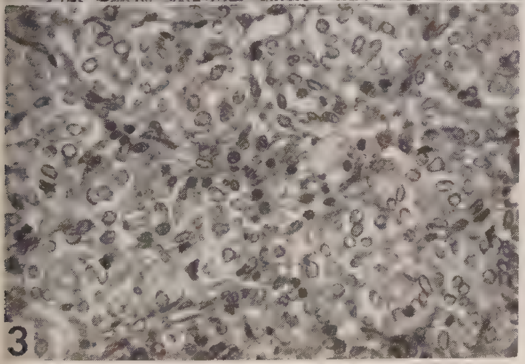


FIG. 1. Thyroid gland, guinea pig. Control animal treated with Freund's adjuvant. Hematoxylin and eosin. × 60.

FIG. 2. Thyroid gland guinea pig. Immunized with a course of 500 μg picrylated thyroglobulin in Freund's adjuvant over 17 days. Alteration of normal architecture as a result of collapse of follicles and apparent loss of colloid. Graded as (++). Hematoxylin and eosin × 60.

FIG. 3. Thyroid gland, guinea pig. Same general field as Fig. 2 in area of follicle collapse. Hematoxylin and eosin × 600.

Were thyroiditis mediated by circulating antibody little or no evidence of a thyroid gland lesion should occur under these conditions. This is in contrast to the actual findings in these experiments.

The lack of a positive correlation between circulating antithyroglobulin antibody and thyroiditis is also apparent from the following data. Whereas only 5% of guinea pigs immunized with 50 μg of picrylated thyroglobulin had detectable circulating antibodies at a low range of titers, 31% of guinea pigs treated with the 10-fold larger immunizing dose exhibited demonstrable circulating antibodies. If thyroiditis had been a direct consequence of antibody formation, the incidence should be much higher in the latter group. However, in both groups the proportion of animals with thyroiditis was the same. Furthermore, among the animals immunized with 500 μg of antigen the percentage of animals with circulating antibodies was almost identical in the groups with and without thyroiditis.

In contrast to the data with respect to circulating antibody, a good positive correlation was noted between delayed type of sensitivity to thyroglobulin and experimental thyroiditis. In both groups of animals, incidence of delayed hypersensitivity as well as incidence of thyroiditis was the same (Table I). The proportion of more severe thyroiditis seemed to be slightly higher in the group immunized with the higher dose, but the difference was relatively small. Table II shows the relationship between thyroiditis and delayed hypersensitivity from another aspect. The proportion of weak skin reactions is particularly high in animals with mild thyroiditis. If one focuses on intensity of the skin reaction as a point of reference, it is interesting to note, that out of 57 animals with a weak skin test, 33 (58%) exhibited thyroiditis, whereas in the group of 19 animals with a strong skin test (marked induration with hemorrhage), 17 (89%) of the animals exhibited thyroiditis.

The percentage of positive skin tests was also quite high in the 45 animals without thyroiditis (62%), although weak reactions were predominant in this group. Does this finding contradict the thesis that delayed hypersensitivity to thyroglobulin is the pathogenetic pathway of experimenal thyroiditis? If this relationship is correct, then the first stage would be the development of delayed hypersensitivity to thyroglobulin and thyroiditis would appear only subsequently. The finding of a relatively high incidence of delayed type of hypersensitivity in animals without thyroiditis is, therefore, consistent with the hypothesis. This raises the question whether a critical level of sensitivity or some additional factor is necessary to elicit the immunologically mediated tissue damage.

Summary. 1. Among each of 2 groups of guinea pigs immunized with 50 μg and 500 μg of picrylated thyroglobulin in Freund's adjuvant respectively, 57.2% (63 of 110 animals) developed thyroiditis 17 days following the initial dose of antigen. Delayed skin reactivity to intradermal thyroglobulin was observed in 72.7% of the animals. There was no significant difference in incidence of thyroiditis and delayed skin reactivity in the 2 groups of animals. However, circulating antithyroglobulin antibody as determined by passive hemagglutination, was found in 4.6% (2 of 43 animals) in the low antigen group and 30% (18 of 60 animals) in the high antigen group.

2. The incidence of thyroiditis and delayed skin thyroglobulin reaction were positively correlated, while no such correlation between thyroiditis and circulating antithyroglobulin antibody was apparent.

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1. Terplan, K. L., Witebsky, E., Rose, N. R., Paine, J. R., Egan, R. W., *Am. J. Path.*, 1960, v36, 213.

2. Roitt, I. M., Doniach, D., Campbell, P. N., Vaughan Hudson, E., *Lancet*, 1956, v1, 820.

3. Rose, N. R., Witebsky, E., Experimental Immunological Thyroiditis, *Immunology, 1st International Symposium*. Ed. P. Grabar and P. Miescher, Publ. Benno Schwabe & Co., Basle, Switzerland, 1958.

4. Witebsky, E., Paine, J. R., and Rosen, N. R., Clinical Significance of Auto-sensitization to Thyroglobulin. *Immunology, 1st International Symposium*. Ed. P. Grabar and P. Miescher. Publ. Benno Schwabe & Co., Basle, Switzerland, 1958.

5. Derrien, Y., Michel, R. J., *Biochem. biophys. Acta*, 1948, v2, 454.

6. Benacerraf, B., and Gell, P. G. H., *Immunology*, 1959, v2, 1, 53.

7. Stavitsky, A. B., *J. Immunol.*, 1954, v72, 360.

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Radiosensitivity of Swine from Irradiated Parentage. (26520)

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Various parameters have been studied in the progeny of irradiated humans(1,2), dogs (3), and rodents(4,5), but we are not aware of any studies on the radiation sensitivity of progeny from irradiated large mammals. Some change in radio-sensitivity might be expected for life shortening effects have been reported in offspring of irradiated mice(6) and rats (7). The present report concerns the radiation response of offspring from parent swine exposed to the mixed gamma-neutron flux from a nuclear detonation.

Material and methods. Offspring from 3 parent categories of predominantly Landrace swine were studied. The *Progeny of Irradiated Parents* group was composed of 89 pigs sired by a boar surviving exposure to 268 rads (55% LD 50/30). The sows had received doses from 268 rads to 515 rads (mean dose 351 rads, 72% LD 50/30). Conditions of exposure for the parent swine have been described(8,9). *Progeny of an Irradiated Male* consisted of 44 offspring resulting from mating an irradiated boar (268 rads) with sows which had been placed as controls during field studies on the effects of a nuclear detonation (8,9). Dosimetric data indicated that these sows received less than 1 rad during the field

TABLE I. Litter Size.

	Avg No. pigs		
Parentage	Per litter	Farrowed alive	Surviving to weaning
Non-irradiated	10.3	9.7	8.0
Irrad. ♂, non-irrad. ♀	11.0	10.5	7.2
Irradiated	10.6	10.4	9.1

test. *Progeny of Non-Irradiated Swine* were 73 pigs produced by non-irradiated parents. Of these, 49 resulted from breeding a mixed breed boar with sows which had served as controls during the field studies mentioned previously. The remainder of the control group (24 pigs) was of similar breed, but from other stock.

The boars were housed separately and first mated with the sows approximately 8 months after irradiation. The first litters were then farrowed about one year after irradiation of the parent animals.

These pigs comprised first and second litters of the sows used. Average litter sizes, number of pigs farrowed/litter, and number of pigs/litter surviving to weaning age based on about 75% of the pigs used (unfortunately some litter size data was lost) is contained in Table I.

The pigs were allowed to eat a 20% protein hog food (without antibiotics) and were well adapted to this food when weaned at 8 weeks of age. The animals were vaccinated against hog cholera and swine erysipelas and were maintained under conditions of accepted swine management. At 11 weeks of age, pigs from each category of parent irradiation were grouped according to the irradiation dose to be received, using a table of random numbers. Physical examinations were performed on all pigs, and weights and hemograms were obtained.

TABLE II. Mortality in Offspring.

	Parentage		
Dose, r	Non-irrad. swine	Irrad. swine (both parents)	Irrad. boar, non-irrad. sow
175	0/8 *	0/14	1/6
225	1/14	0/18	0/9
280	4/14	3/19	5/12
360	8/14	9/18	5/10
450	12/14	14/17	6/7
570	5/5	3/3	—
720	4/4	—	—
LD 50/30	335r	361r	332r
95% confidence limits	303-370r	333-391r	282-390r

* No. dead within 30 days/No. irradiated.

The pigs were irradiated at 84 (±1) days of age with X-rays produced by a 3.5 mev Van de Graaff generator operated at a constant potential with a dose rate of approximately 150 r/min. The radiation source and calibration procedures used have been described(10). After irradiation the swine were fed commercial hog food (without antibiotics) and water was available *ad libitum*.

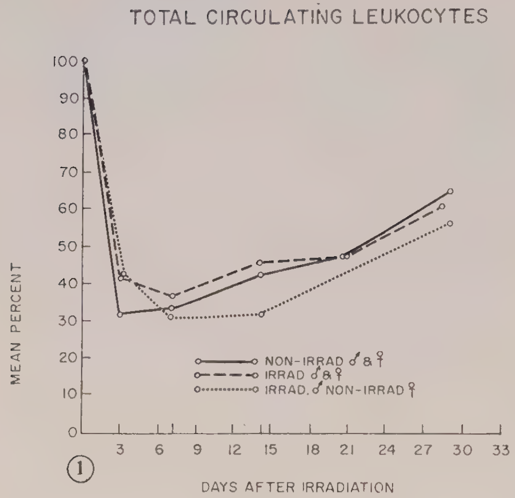
TABLE III. Survival Time, Days.

	Parentage					
	Non-irradiated		Irradiated		Irrad. ♂, non-irrad. ♀	
Dose, r	Mean	Range	Mean	Range	Mean	Range
175	—	—	—	—	21	21
225	21	21	—	—	—	—
280	19	17–21	17	14–20	15	15–17
360	17	11–22	14	6–19	18	12–27
450	14	9–21	14	9–26	14	10–15
570	11	9–15	8	7– 9	—	—
720	9	7–10	—	—	—	—

Results. The number of pigs used and mortality in each group is contained in Table II for various experimental doses. By probit analysis(11) an LD 50/30 of 335r for offspring of non-irradiated swine was obtained. For progeny of irradiated parents LD 50/30 was 361r, and for pigs from the irradiated boar and non-irradiated sows LD 50/30 was 332r. Ninety-five per cent confidence limits were similar for all groups. The radiation response of offspring from the most heavily irradiated parents (mean dose to sows 460 rads, 95% LD 50/30, dose to boar, 268 rads) did not differ from that of the control population.

Mean survival times (Table III) did not differ significantly for the 3 categories of parent irradiation at different dose levels, or when all decedents in each parent category were combined. These survival times are dose dependent and are comparable to those usually reported for midlethal range irradiation.

Mean circulating leukocyte response and



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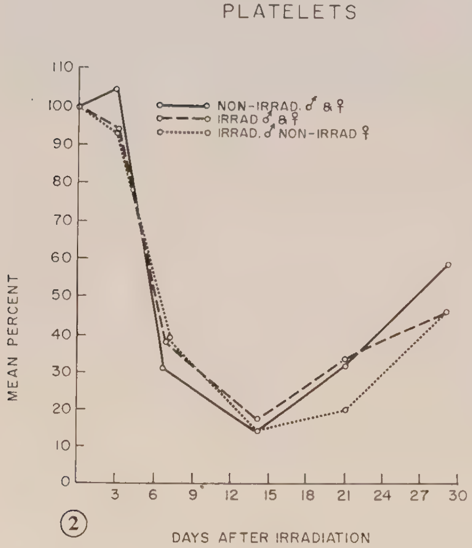

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FIG. 1. Circulating leukocyte response for all swine. Plotted points are mean % of mean pre-irradiation values.

FIG. 2. Blood platelet response for all swine. Points are mean % of pre-irradiation values.

mean platelet response for all swine in each category of parent irradiation are presented in Fig. 1 and Fig. 2, respectively.

Times of onset and signs and symptoms of the radiation syndrome, as well as weight response for each group were as expected for midlethal range irradiation. Gross pathological changes seen at autopsy were typical of radiation damage and related to survival time.

Discussion. The failure to observe a change in radiation sensitivity could have resulted from our inability to optimize experimental conditions. Mouse data(6) indicate that for genetic effects neutrons are more effective than photons, and that maximal effects are to be expected from matings 19-23 days after irradiation. Both the delayed breeding time of 8 months and the fact that the radiation of the parents was about 50% gamma radiation and 50% neutrons operate to reduce the expected effect. Comparative data are not available on radiation effects of offspring of irradiated females. If effects on male and female are additive an enhanced effect would be expected in our irradiated parent group.

The decrease in life span following sublethal irradiation has been related to a decrease in radiation resistance(12,13,14). Other workers have failed to show such a decrease in resistance to radiation after recovery from an acute radiation exposure(15,16). From our data it appears that the point estimate of 0.077% life shortening for each rep received by the paternal parent(6) cannot be directly applied to a decrease in the LD 50/30 in progeny produced some time after irradiation of large mammals.

A brief comment on litter size shown in Table I is worthwhile. Generalizations cannot be made from these particular matings. However, there was no indication that irradiation produced a reduction in litter size or in viability of the offspring. Litter sizes were similar for all groups and number of pigs surviving to weaning age is consistent with the experimental facilities. Our data agree with that reported for X-irradiated dogs(17). The relatively low average for pigs surviving to weaning age in the irradiated boar—normal sow group is due to one litter in which a sow having insufficient milk saved only 3 of 15 live pigs farrowed.

Demonstration of a decrease in litter size from irradiation of the paternal parent to this dose would not be expected from the number of matings reported herein. However, the continued fertility of these sows with no decrease in litter size after surviving approximately 55-106% LD 50/30 acute dose and the similar results in dogs(17) are in distinct contrast to the results in mice in which similar doses have produced drastic effects on fertility.

Two hundred and six 12-week-old swine from non-irradiated parents, from irradiated parents, and from an irradiated boar and non-irradiated sows showed no significant difference in sensitivity to X-rays as measured by 30-day lethality. Lethality was essentially identical for each group as was survival time, hematological response, weight change, clinical signs of radiation sickness and gross changes at autopsy.

Summary. LD 50/30 values for 12-week-old offspring from non-irradiated swine, from irradiated swine, and from an irradiated boar and non-irradiated sows were not significantly different. Survival times for progeny of the different parent categories were not significantly different and were within the usual range for midlethal irradiation. Other responses were similar in each group.

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1. Miller, R. W., *Pediatrics,* 1956, v18, 1.

2. Neel, J. V., Schull, W. J., Nat. Acad. Sci., Nat. Research Council, Washington, D. C., 1956.

3. Rekers, P. E., *J. Lab. Clin. Med.,* 1951, v37, 331.

4. Spalding, J. F., Hawkins, S. B., Strang, V. G., *Radiation Research,* 1958, v8, 222.

5. ———, *ibid.,* 1958, v9, 369.

6. Russell, W. L., Proc. Nat. Acad. Sci., 1957, v43, 324.

7. McGregor, J. F., James, A. P., Newcombe, H. B., *Radiation Research,* 1960, v12, 61.

8. Woodward, K. T., McDonnel, G. M., Harris, P. S., Kirkland, W. J., Shively, J. N., *Am. J. Roent. Rad. Ther. Nuc. Med.,* 1961, v85, 179.

9. McDonnel, G. M., Claypool, H. A., Moncrief, W. H., Goldstein, J. D., Project 4-1, ITR-1428, AEC Technical Information Service, Oak Ridge, Tenn.

10. Andrews, H. L., *Radiation Research,* 1958, v9, 469.

11. Finney, D. J., Probit Analysis. 2 ed., Cambridge Univ. Press, 1952.

12. Blair, H. A., Univ. Rochester Rep. UR-442, 1956.

13. Hursh, J. B., Van Slyke, F., Casarett, G. W., Univ. Rochester Rep. UR-318, 1954.

14. Hursh, J. B., Noonan, T. R., Casarett, G. W., Van Slyke, F., *Am. J. Roent. Rad. Ther. Nucl. Med.,* 1955, v 74, 130.

15. Sacher, G. A., Argonne Nat. Lab. Rep., ANL-4163, 1948, 83.

16. Storer, J. B., *Radiation Research,* 1959, v10, 180.

17. Anderson, A. C., Schultz, F. T., *ibid.,* 1960, v12, 417.

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Interaction of Histamine, Serotonin and Heparin with Hexadimethrine Bromide, a Mast Cell Fragmentor. (26521)

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We reported(1,2) that hexadimethrine bromide (Polybrene*), an anti-heparin agent, caused disruption of tissue mast cells in the rat both *in vitro* and *in vivo.* It was suggested that histamine, serotonin and heparin, presumably released from these fragmented tissue mast cells, might perhaps be factors contributing to the symptomatology of this polymer following its injection in large doses.

This present study was undertaken to explore further the possible role of these mast cell components as they affect the toxicologic response to this anti-heparin agent in mice, rats and guinea pigs. To this end, experiments were run to ascertain whether depletion or accumulation of either or both of these amines by prior pretreatment with suitable agents could alter the subsequent response of the animals to a lethal dose of this polymer.

Methods. To deplete most of the tissues of the rat of their histamine, 5 injections of polymyxin B sulfate[†] were given intraperitoneally over a 3-day period according to the method of Parratt and West(3). Animals weighing between 150-180 g were injected with a single dose of 2.5 mg/kg of polymyxin B on the 1st day, 2 doses of 5 mg/kg on the 2nd day and 2 doses of 7.5 mg/kg on the 3rd day. On the fourth day, the animals were divided into groups of 10 and injected intravenously with various dose levels of hexadimethrine. Controls were run concurrently on saline treated rats here and in all subsequent tests; all survivors were observed for 1-2 weeks.

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* 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide, Abbott.

An intraperitoneal injection of reserpine, 10 mg/kg, was given to rats (160-180 g) to deplete most of their tissues of their serotonin (4). Hexadimethrine in graded doses was given to groups of these rats 24 hours after reserpinization in order to establish its LD_{50} following this pretreatment.

Rats weighing between 90-110 g were injected intraperitoneally twice daily for 5 days with compound 48/80 to deplete the tissue mast cells of the rat of their histamine, heparin and possibly serotonin(4,5). The initial dose on the 1st day was 100 μg/rat; the doses were then increased by increments of 100 μg each day to reach a dose of 500 μg by the 5th day. On the 6th day, the animals were divided into groups of 10 and intravenous LD_{50} determined for hexadimethrine.

To simulate, in part, the pharmacologic responses of the animals toward 48/80, groups of rats were pretreated with both polymyxin B sulfate and reserpine prior to hexadimethrine. Pretreatment regimens for both drugs were as described previously, except that one less injection of polymyxin was given to these animals.

To test the possibility of histamine potentiation by hexadimethrine, groups of mice weighing between 20-22 g were pretreated for 1 hour with either 2 or 6 mM/kg of imidazole, subcutaneously. Animals were then divided into groups of 20 and given hexadimethrine, intraperitoneally, to establish its comparative LD_{50} following this pretreatment regimen to inhibit diamine oxidase, *i.e.*, histaminase. Thus, several fixed doses of the inhibitor were pitted against the dose-mortality curve of hexadimethrine.

A similar pretreatment regimen was followed in mice using semicarbazide, another diamine oxidase inhibitor. Doses of 12.5 and 50 mg/kg were given subcutaneously to groups of mice 30 minutes prior to hexadimethrine given intraperitoneally at various dose levels.

As an ancillary study, a second series of mice was pretreated further with 10 and 25 mg/kg of chlorcyclizine hydrochloride, orally, 1 hour prior to imidazole. The rationale underlying this multi-pretreatment schedule stemmed from the hypothesis that the antihistaminic agent would perhaps counteract the histamine released by hexadimethrine and protected from inactivation by imidazole. Thus, a subsequent injection of hexadimethrine would perhaps be able to exert its effects without the histamine component entering the picture.

Using a specie more susceptible to histamine, groups of guinea pigs, 200-300 g, were pretreated, intraperitoneally, with 10 and 24 mg/kg of chlorcyclizine, 90 minutes prior to being pitted against the dose-mortality curve of hexadimethrine, intravenously. A second series of animals was pretreated for 4 hours with 15 mg/kg of the antihistaminic, intraperitoneally, then challenged with a lethal dose (22 mg/kg) of hexadimethrine, intravenously.

As an ancillary experiment, mice (20-22 g) in groups of 10 were pretreated intravenously with varying doses of either heparin sodium (150 U.S.P. units/mg) or with "inactivated" heparin[‡] to compare their prophylactic potencies against the anti-heparin agent given at a fixed dose of 56 mg/kg, intravenously, equivalent to 2 × LD_{50}.

The molecular weight of the hexadimethrine used in this study was 6000.

Male Scientific strain mice and male Holtzman rats were used in all these studies. The

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[†] Burroughs Wellcome & Co., Tuckahoe, N. Y.

[‡] Batch #1582-82, "inactivated" by boiling in 0.08 N HCl for 3 hours and containing only 2.5-3 U.S.P. units/mg.

TABLE I. Effect of Depletion of Tissue Amines on Hexadimethrine Bromide Toxicity in Rats, I.V.

Pretreatment drugs*	Hexadimethrine bromide LD_{50} in mg/kg (95% C.L.) Controls (A)	Pretreated (B)	Toxicity ratio $= \frac{B}{A}$ (95% C.L.)
48/80	19.5 (17.4-21.8)	26.0 (22.0-30.0)	1.33† (1.11-1.60)
Polymyxin B	20.0 (17.2-23.2)	24.5 (20.4-29.4)	1.23 (.98-1.53)
Reserpine	20.0 (17.3-22.8)	23.0 (21.7-24.4)	1.15 (1.00-1.32)
Polymyxin B + reserpine	22.8 (20.5-25.3)	18.5 (14.5-23.7)	1.23‡ (.95-1.59)

* See text for dosage regimen. † Significantly different. ‡ Toxicity ratio of $\frac{A}{B}$.

method of Litchfield and Wilcoxon(6) was used for all statistical calculations.

Results. Table I shows that pretreatment of rats with either polymyxin B or with reserpine failed to alter significantly the subsequent LD_{50} values for hexadimethrine obtained in these same animals. Toxicity ratios were 1.23 and 1.15 for polymyxin B and for reserpine pretreatments, respectively. However, pretreatment with 48/80, which is reported to deplete both amines, significantly decreased the toxicity of hexadimethrine given subsequent to this prophylaxis. The intravenous LD_{50} for hexadimethrine controls in this series of experiments was 19.5 mg/kg while LD_{50} value for 48/80 pretreated animals given the anti-heparin agent was 26 mg/kg.

Concomitant pretreatment with polymyxin B and reserpine did not, however, simulate the prophylactic response obtained with 48/80 pretreatment alone. On the contrary, there was increased sensitivity towards hexadimethrine in these animals when it was given after the dual chemical assault by these 2 pretreatment agents. The LD_{50} value for hexadimethrine in this series of experiment was 22.8 mg/kg while that for the multi-pretreated group was 18.5 mg/kg. That this dual pretreatment regimen may have yet shown a subtle protective effect was inferred from several observations. There was absence of the characteristic edema of the extremities as induced by hexadimethrine; onset of death was delayed up to 7 days in the pretreated group while deaths occurred during the second through the fourth days in the control groups, albeit final mortality ratio was greater with the pretreatment.

The data in Table II show that subcutaneous pretreatment of mice with imidazole at a dose level of 2 mM/kg, equivalent to one-sixth of its LD_{50} (and to LD_0), did not materially affect hexadimethrine bromide toxicity. Increasing the pretreatment dose to 6 mM/kg, equivalent to one-half LD_{50} (and to $LD_{0.5}$), significantly increased the sensitivity of the animals toward a subsequent dose of the anti-heparin agent. Thus, control animals showed a LD_{50} value of 46 mg/kg while the LD_{50} value for imidazole pretreated mice was about one-half, *i.e.*, 22.5 mg/kg. Pretreatment of mice with semicarbazide, another diamine oxidase inhibitor, at dose levels of 12.5 and 50 mg/kg did not alter the dose mortality curve of hexadimethrine.

Pretreatment of mice with 10 and 25 mg/kg of chlorcyclizine, equivalent to about 1/25 and 1/10 of its LD_{50}, caused a slight antagonistic action towards the toxicogenic effect of imidazole on hexadimethrine. This was more evident with the lower dose of the antihistaminic wherein pretreatment with 10 mg/kg caused a slight decrease in the imidazole induced toxicity of hexadimethrine from a value of 22.5 mg/kg to 34 mg/kg. However, the magnitude of this protective effect was not pronounced.

Again, intraperitoneal pretreatment for a period of 90 minutes with 10 and 24 mg/kg of chlorcyclizine did not decrease hexadimethrine toxicity in guinea pigs. Rather, an indication of additive toxicity was evident at the higher dose of chlorcyclizine. Furthermore, intraperitoneal pretreatment for 4 hours with 15 mg/kg of chlorcyclizine had no protective effect against a lethal intravenous dose of hexadimethrine.

TABLE II. Effect of Imidazole and Chlorcyclizine Pretreatments on Hexadimethrine Bromide Toxicity.

Pretreatment drugs	mM/kg, s.c.	mg/kg	Hexadimethrine bromide LD_{50} in mg/kg (95% C.L.) Controls (A)	Pretreated (B)	Toxicity ratio = $\frac{A}{B}$ (95% C.L.)
A. In mice:				(Intraperitoneal)	
Imidazole	2		46.0 (42.8-49.5)	41.0 (37.3-45.1)	1.1 (1.0-1.3)
"	6*		46.0 (42.8-49.5)	22.5 (19.1-26.6)	2.0† (1.7-2.4)
Chlorcyclizine + imidazole	6	10-oral	48.0 (45.7-50.4)	34.0 (31.5-36.7)	1.4 (1.3-1.6)
Chlorcyclizine + imidazole	6	25-oral	48.0 (45.3-50.9)	25.0 (22.7-27.5)	1.9† (1.7-2.1)
B. In guinea pigs:				(Intravenous)	
Chlorcyclizine		10‡	18.8 (18.1-19.6)	18.5 (17.7-19.4)	1.0 (.9-1.1)
"		24	18.8 (18.1-19.6)	14.8 (13.4-16.3)	1.3 (1.2-1.4)

* Non-lethal *per se* and equivalent to about $LD_{0.5}$. LD_{50} and 95% C.L. of imidazole determined to be 11 mM/kg (10.2-11.8).

† Significantly different.

‡ Intraper. dose which protects against about 25 lethal doses of histamine, intravenously.

Table III shows that "inactivated" heparin, containing only 2.5-3 U.S.P. units/mg of heparin, was only half as potent as "regular" heparin sodium (150 U.S.P. units/mg) as a prophylactic agent against hexadimethrine given at a dose level of twice its LD_{50}.

None of the comparative LD_{50} slopes showed any significant deviation from parallelism (Tables I and II).

Discussion. The failure of combined pretreatment with polymyxin and reserpine to decrease hexadimethrine toxicity may be explained on the basis of additive toxicity from these 2 pretreatment drugs. Reserpinization of rats already subjected to polymyxin pretreatment caused a further debilitation in these animals. In contrast, pretreatment with a single drug depleting both amines, namely, 48/80, actually increases the resistance against hexadimethrine.

A relatively high dose of imidazole was required to exert significant effect on hexadimethrine toxicity (Table II). That the resultant toxicity ratio was only of the order of 2.0 would be in accord with previously documented findings that histamine does not play a major role in rodents as it does in some other species.

TABLE III. Comparative PD_{50} Values of Heparin Sodium and "Inactivated" Heparin *vs* 2 × LD_{50} of Hexadimethrine Bromide, I.V. (56 mg/kg) in Mice.

Pretreatment drug	Calculated PD_{50}, I.V., (mg/kg) *vs* 2 × LD_{50} hexadimethrine, I.V. (56 mg/kg)
Heparin sodium	52
"Inactivated" heparin	90

Results obtained with imidazole and semicarbazide pretreatment would tend to support the data of Angelakos and Loew(7) that of a large series of histaminase inhibitors, only imidazole was capable of significantly potentiating histamine toxicity in mice.

The finding that an antihistaminic was of little or no value against hexadimethrine toxicity in guinea pigs and mice substantiated our earlier findings(1,2) that pretreatment with antihistaminic agents was able only partially to alter the character of the hypotensive response to hexadimethrine in dogs. MacIntosh and Paton(8) have pointed out that although antihistaminic drugs are able to antagonize the circulatory effect of histamine liberators, their antagonism is only of a limited nature.

Previous experiments(4,9,10) have shown that a pretreatment regimen with 48/80, polymyxin and reserpine can deplete most of the tissues of the rat of their histamine and/or serotonin. Our previous reports(1,2) showed hexadimethrine capable of fragmenting tissue mast cells in the rat both *in vivo* and *in vitro*. If the symptomatology observed in animals given high doses of this polymer is due, in part, to the action to these amines, then animals previously depleted of these amines would be less sensitive to the action of a drug which presumptively acted *via* this pathway. Such may be the case with hexadimethrine.

Summary. Animals partially depleted of their histamine and serotonin by pretreatment with 48/80 were rendered less sensitive to the toxic effects of hexadimethrine. Conversely, pretreatment of mice with imidazole increased the toxic effects of a subsequent injection of hexadimethrine. These results, coupled to our previous findings(1,2) suggest that some of the responses to hexadimethrine in certain laboratory animals may be mediated *via* histamine, serotonin and heparin release. It is probable that there are other factors which may be concerned in the over-all toxicologic and pharmacologic picture of this antiheparin agent.

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1. Kimura, E. T., Young, P. R., Stein, R. J., Richards, R. K., *Tox. & Appl. Pharmacol.*, 1959, v1, 185.
2. Kimura, E. T., Young, P. R., Ebert, D. M., *ibid.*, 1959, v1, 560.
3. Parratt, J. R., West, G. B., *J. Physiol.*, 1957, v137, 179.
4. West, G. B., *Int. Arch. Allergy*, 1957, v10, 257.
5. Riley, J. F., West, G. B., *Arch. int. Pharmacodyn.*, 1955, v102, 304.
6. Litchfield, J. T., Jr., Wilcoxon, F., *J. Pharmacol. & Exp. Therap.*, 1949, v96, 99.
7. Angelakos, E. T., Loew, E. R., *ibid.*, 1957, v119, 444.
8. MacIntosh, F. C., Paton, W. D. M., *J. Physiol.*, 1949, v109, 190.
9. Telford, J. M., West, G. B., *J. Pharm. & Pharmacol.*, 1960, v12, 254.
10. Bhattacharya, B. K., Lewis, G. P., *Brit. J. Pharmacol.*, 1956, v11, 411.

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Diphosphopyridine Nucleotide Binding Effect of Sickle Cell Erythrocytes On *in vitro* Growth of *Hemophilus influenzae*.* (26522)

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The recent demonstration that erythrocytes from patients with sickle cell anemia fail adequately to support *in vitro* growth of certain hemophilic bacteria(1) suggests the existence of an unidentified inhibitor in these erythrocytes. The present investigations demonstrate and identify the inhibitory system.

Materials and methods. Blood from 8 patients with sickle cell anemia, 6 patients with sickle cell trait, and 5 normal controls was collected under sterile conditions in standard (A.C.D.) anticoagulant. The blood cells were immediately washed 3 times with sterile 0.85% saline solution. The washed cells were resuspended in physiological saline to a hematocrit value of 40% and incorporated in culture media.

Trypticase soy agar and broth (BBL) was employed for agar plates and broth cultures. Erythrocyte suspensions were added to the cultures in a concentration of 4%.

Lyophilized cultures from the American Type Culture Collection, Washington, D.C., were used: *Hemophilus influenzae* type *A* (9006); *H. influenzae* type *B* (9334); *Diplococcus pneumoniae* type *III* (6303); and *Streptococcus salivarius* (9756). Hospital laboratory cultures of *H. influenzae* type *A; H. influenzae* type *B*; *Salmonella montevideo*; *S. oranienberg*; and *S. bareilly* were also employed.

Inocula were prepared from 24 hour broth cultures. A single 0.04 ml drop of a 1:200 di-

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* Aided by a grant from Otho S. A. Sprague Memorial Inst. Laboratories, Children's Memorial Hospital.

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lution of the broth culture was placed in the center of each Petri plate and was spread with a sterile glass stirring rod. Ten replicate platings were made each time the growth of the organism was studied. Plates were observed and growth recorded at 24 hours. Tube broth cultures containing 10 ml of media were inoculated with 0.04 ml of a 1:200 dilution of a 24 hour broth culture and growth recorded at 24 hours. The size of surface colonies was estimated as + (barely visible) to ++++ (larger than 1 mm in diameter). Since replicate inocula resulted in almost exactly the same number of colonies (100), no report of colony numbers was necessary. Growth of broth cultures was estimated as + (barely visible) to ++++ (heavy turbidity).

Chocolating of erythrocytes was carried on at 80°C. Peptic digestion was done according to the method of Fildes(2). Osmotic fragility and alkali denaturation were determined by the methods of Chernoff(3). Diphosphopyridine nucleotide (Assay 95%) was purchased from Sigma Chemical Co.

Results. Preliminary screening studies were conducted with surface plate cultures containing washed erythrocytes from patients with sickle cell anemia (SS), sickle cell trait (SA) and normal adult blood (AA). Representative organisms of the genus *Hemophilus, Diplococcus, Streptococcus,* and *Salmonella* were cultured. *H. influenzae* type *A* and *B* organisms grown on media containing SA or AA erythrocytes grew well (+++ or ++++), however, when grown on media with SS erythrocytes, growth was poor (+). No similar differential growth pattern was consistently found with the other microorganisms. The observations with the genus *Hemophilus* were confirmed with blood samples from several different patients and with freshly isolated cultures. Identical results were found when blood samples were defibrinated instead of being treated with anticoagulant. Because of this inhibition of growth on media containing SS erythrocytes, all further investigations were conducted with the *H. influenzae* type *B* organism.

The physical-chemical properties of representative samples of SS, SA and AA erythrocyte suspensions were determined. Resistance to hemolysis in hypotonic salt solutions was greatest for SS erythrocytes and least for AA erythrocytes. Microscopic examination of agar and broth culture media containing washed erythrocytes showed no greater hemolysis for SS than SA and AA erythrocytes. Resistance to alkali denaturation was greatest for SS erythrocytes (5-7%) and least for SA and AA erythrocytes (3.2-3.8%).

The loss of the ability of the SS, SA, and AA erythrocytes to support the growth of *Hemophilus* organisms under various conditions was studied with the surface plate technic. Samples of each type of washed erythrocyte suspension were permitted to stand both at room temperature and at 0°C for variable periods of time before being incorporated into culture medium. It was found that while SS erythrocytes were capable of supporting minimal (+) bacterial growth when immediately incorporated into media, this capacity disappeared completely if the erythrocytes were permitted to stand one hour or more before being added to the media. Similarly, SA and AA erythrocytes completely lost their ability to support growth if permitted to stand 6 hours or more. Hemolysis of any of the 3 types of erythrocytes by hypotonic salt solution, shaking, freezing, Meckle disintegration, or sonic oscillation immediately eliminated the bacterial growth supporting potential.

Methods of protecting and enhancing the ability of the SS, SA, and AA erythrocytes to support growth of *Hemophilus* organisms were demonstrated in several ways by the surface plate technic. Immediate chocolating of the washed erythrocyte suspension resulted in ++++ growth of the microorganisms for SS as well as SA and AA preparations. This was true even when several hours elapsed between the time of chocolating and incorporation of the material in the media. Peptic digestion of SS, SA, or AA erythrocytes as long as 5 days after the original preparation of the suspension resulted in ++++ growth of the microorganisms in each case. Furthermore, peptic digestion completely restored the growth supporting ac-

TABLE I. Stimulation of Growth of *H. influenzae* Type *B* by Treated Suspensions of Erythrocytes.

Conc. of erythrocyte suspension†	Erythrocyte suspensions*					
	Immediate chocolating			Peptic digestion		
	SS	SA	AA	SS	SA	AA
1:250	++++	++++	++++	++++	++++	++++
1:500	++++	++++	++++	++++	++++	++++
1:1000	+++	+++	+++	+++	+++	+++
1:2000	++	++	++	++	++	++
1:4000	+	+	+	+	+	+
1:8000	0	0	0	0	0	0
1:16000	0	0	0	0	0	0

* Washed erythrocytes, growth stimulating activity of which had been inactivated by standing 6 hr. Bacterial growth 0 to ++++.

† Concentration in terms of original erythrocyte suspension after addition to 10 ml of broth—equivalent in chocolated erythrocytes or peptic digestate. Hemin (1 mg) added to each culture tube.

tivity of inactive (hemolyzed) SS, SA, or AA erythrocytes so that media prepared with any of these preparations resulted in ++++ bacterial growth. Table I reveals that restoration of activity with peptic digestion was equal to that activity found with immediate chocolating of the erythrocyte suspensions. Erythrocyte stroma prepared from hemolyzed SS, SA, and AA erythrocytes by washing 3 times with saline, filtering, and peptic digestion were similarly capable of supporting ++++ bacterial growth while the supernatant of preparations so treated was inactive. Addition of DPN 1 mg% with or without Hemin 10 mg% to fresh, old or hemolyzed suspensions of SS, SA or AA erythrocytes resulted in ++++ bacterial growth. Table II demonstrates the greater binding effect of SS erythrocytes when compared to SA and AA erythrocytes.

TABLE II. Inhibition of Growth of *H. influenzae* Type *B* through DPN Binding by Erythrocytes.

DPN added (μg)	Erythrocyte suspension*		
	SS	SA	AA
0	0	0	0
10	0	+	+
20	0	++	++
40	0	+++	+++
80	+	++++	++++
100	++	++++	++++
200	+++	++++	++++
400	++++	++++	++++
800	++++	++++	++++

* Washed erythrocytes, growth promoting activity of which had been inactivated by standing 6 hr. Bacterial growth 0 to ++++.

DPN added 2 hr before suspension was introduced into broth.

Discussion. The results of the experiments demonstrate that DPN required for growth of *H. influenzae* type *B* is normally present in SS, SA, and AA erythrocytes in amounts sufficient to support heavy bacterial growth when added to suitable media. However, in the case of SS erythrocytes DPN is almost completely removed shortly after the sample is obtained from the patient and is totally unavailable after standing for one hour. DPN is initially available from SA and AA erythrocytes in quantities capable of stimulating heavy bacterial growth but becomes completely bound in 6 hours. Differnces in availability of DPN would appear to be associated with the greater binding capacity demonstrated to be present in SS erythrocytes.

The site of binding is the stroma of the erythrocyte. Immediate chocolating of any of the fresh preparations alters the erythrocytes so that binding is prevented. The bound DPN can be completely released by peptic digestion of the preparation. Only minimal quantities of bound DPN are released by chocolating and only from the SA and AA erythrocytes. Hemolysis greatly augments binding, apparently by exposing stromal sites of binding. Spontaneous hemolysis however does not appear to be responsible for the increased binding of DPN by SS erythrocytes as these cells are more resistant to hemolysis by hypotonic salt solutions and they show no greater number of hemolyzed erythrocytes when observed directly in the culture medium. Interestingly, incorporation of SA and AA erythrocytes into culture media apparently inhibits binding of DPN sufficiently to permit release for bacterial growth.

Inactivation of DPN (V factor) in blood of animals has been discussed by Lwoff and Lwoff(4). These observations were extended to include several animal species by Krumwiede and Kuttner(5). Further information comparing various species was reported by Waterworth(6). These observations have lead to the hypothesis that DPN is enzymatically destroyed(6) and evidence for a DPN splitting enzyme has been reported by Denstedt(7) and others.

The information reported here concerning the effect of human SS, SA, and AA erythrocytes on growth of *H. influenzae* type *B* does not appear compatible with the hypothesis of a DPN destroying enzyme. Instead, the presence of a system of reversible erythrocyte stromal binding of DPN is supported by the fact that: 1. Hemolysis immediately removes DPN, 2. Chocolating prevents removal of DPN, and 3. Peptic digestion completely releases DPN. The differences between SS erythrocytes and SA or AA erythrocytes are due to the more rapid and extensive binding of DPN which occurs with the SS cells. The actual mechanism of the bond requires further investigation. Preliminary experiments suggest that the binding is not enhanced, retarded or reversed by the presence of human albumin or pH changes between 4.5 and 8.5.

Summary. Experiments are reported which demonstrate that DPN required for growth of *H. influenzae* type *B* is normally present in equal amounts in erythrocytes from patients with sickle cell anemia, sickle cell trait, and normal adults. This material disappears within one hour from sickle cell anemia erythrocyte preparations and in 6 hours from sickle cell trait and normal adult erythrocyte preparations. The difference in rate of disappearance is caused by the greater DPN binding capacity of sickle cell anemia erythrocytes. Hemolysis stimulates binding of DPN; chocolating prevents binding; and peptic digestion frees bound DPN. The site of the bond is the stroma of the erythrocyte.

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1. McCormick, W. F., Flanigan, C., Francisco, T. J., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 847.
2. Fildes, P., *Brit. J. Exp. Path.*, 1920, v1, 129.
3. Chernoff, A. I., *New England J. Med.*, 1955, v253, 322.
4. Lwoff, A., and Lwoff, M., *Proc. Royal Soc. London*, 1937, v122, 352.
5. Krumwiede, E., Kuttner, A. G., *J. Exp. Med.*, 1938, v67, 429.
6. Waterworth, P. A., *Brit. J. Exp. Path.*, 1955, v36, 186.
7. Malkin, A., Denstedt, O. F., *Canad. J. Biochem. & Physiol.*, 1956, v34, 141.

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Studies of the Hemagglutinins of Type 3, 4 and 7 Adenovirus. (26523)

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Rosen(1) showed that adenoviruses agglutinate erythrocytes of various species of animals selectively. The following work was initiated, in part, to confirm Rosen's observations and to study the properties of the hemagglutinin of Type 3 adenovirus (ADV-3). An attempt was made also to determine whether or not the hemagglutinin component of the adenovirus is a soluble substance.

Materials and methods. Virus. Viruses used in these studies were Types 3, 4, and 7 adenoviruses (ADV-3, 4, and 7) which were originally obtained from Dr. Heubner's laboratory. These viruses can be grown in either monkey kidney (MK) epithelial or HeLa cells. Most virus preparations were made in 1-liter or 5-liter Blake bottles containing confluent sheets of MK cells. Virus was harvested when cellular degeneration was complete, usually 5 to 7 days' incubation at 37°C. All virus pools used were prepared in absence of serum and stored at 4°C.

Concentration—Virus concentrates were prepared by methods described by Prier and LeBeau(2).

Antisera. Rabbit antisera against various types of adenovirus were prepared according to methods described by Rowe, *et al.*(3). Virus preparations used for immunizing rabbits were clarified by either low speed centrifugation or filtration through Seitz clarification pads to remove excessive cullular debris.

Hemagglutination (HA) and Hemagglutination Inhibition (HI) Test—HA and HI tests were performed according to procedures outlined by Rosen(1), using as routine, erythrocytes obtained from the grivet or green monkey (*Cercopithecus aethiops*). Applicability of rhesus and guinea pig cells for these tests were also compared.

Complement fixation (CF) test. Concentrations of complement-fixing antigens were determined by the technic described by Osler, Straus, and Mayer(4), using the 50% hemolytic endpoint.

Centrifugation studies. All centrifugations were made with a No. 40 roter in the Model L Spinco at 4°C at speeds and times indicated.

Filtration studies. Filtrates were prepared by passing a minimum of 15 liters of harvested material through a Seitz-type, 6-inch diameter sterilizing pad. Sediment of harvested material was left undisturbed in the bottom of the glass container to prevent clogging of pads.

Separation of HA from CF activity by adsorption with Grivet Erythrocytes. Washed and packed erythrocytes were added to virus preparations to obtain a final concentration of 2%. Mixtures were agitated with a magnetic stirrer for 45 minutes at room temperature to keep the red blood cells in suspension. The mixture was then centrifuged at 2500 rpm for 5 min. Supernates were drawn off and subjected to the same procedure with fresh red blood cells. This cycle was repeated 3 times. Red blood cells were pooled, diluted 1:10 in distilled water. The cell suspension was frozen and thawed twice to lyse the cells. Lysates, as well as supernates, were tested for CF and HA activity.

Results. The data in Table I confirmed the results of Rosen(1) that Type 3 adenovirus (ADV-3) agglutinates erythrocytes obtained from either rhesus or grivet monkeys. Erythrocytes of the grivet were more sensitive to agglutination than rhesus erythrocytes. Adenovirus suspensions prepared from HeLa cells seemed to contain a greater concentration of hemagglutinin than those prepared from MK cells.

ADV 4 was also capable of agglutinating erythrocytes of green monkeys (Tables II and III). This finding was at variance with results obtained by Rosen(1). The difference may be due to individual variation of monkeys used as blood donors.

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TABLE I. HA Titration of ADV-3 Using Erythrocytes from Different Animals.

Virus preparation	Tissue culture source	HA titers* with erythrocytes from		
		Guinea pig	Grivet monkey	Rhesus monkey
ADV-3	MK†	<4	16	8
" (100 × conc.)	MK	<4	512	64
"	HeLa	<4	2048	64
Control	MK	<4	<4	<4
"	HeLa	<4	<4	<4

* Titer expressed as reciprocal of highest dilution of virus in 0.5 ml of saline showing complete agglutination.

† MK—Rhesus monkey kidney cells.

Hemagglutinin of ADV-3 was fairly stable, (Table II). Activity was not completely destroyed by 1:4000 formalin and/or by storage at 4°C for as long as 2 years. As shown in the same table, hemagglutinins derived from ADV-4 and ADV-7 were also stable after storage at 4°C for over a year. Although original materials were not available for testing at the time of this study, HA titers of fresh MK-grown virus ranged from 64 to 128, and titers of fresh HeLa-grown virus, from 256 to 1024. Since these titers were about the same as HA titers of the adenoviruses mentioned in Table II, it is safe to assume that there had been no reduction of these titers after prolonged storage.

Attempts were made to follow inactivation of HA activity of ADV-3 at 56°C. HA titer dropped from 256 to 32 after 10 minutes at this temperature and remained constant (16 to 32) for the next 40 minutes. This indicated that either HA activity was moderately heat stable or that 2 types of hemagglutinins might be present in the preparation; one labile and one stable at 56°C.

Results of centrifugation studies shown in Table III indicated that HA activity was not sedimentable at speeds of 20,000 rpm for 60 minutes. However, at speeds of 36,000 to 39,000 for 90 to 180 minutes, HA activity was sedimented while most of the CF antigen usually remained in the supernatant fluids. These results indicated that the HA was a soluble component but different from the CF component. This difference was further emphasized in filtration studies. Data in Table IV showed that CF factor was partially removed by Seitz (S-1) sterilizing pads, whereas HA fractions appeared undiminished in filtrates.

Type specific rabbit antisera were prepared against adenoviruses, Types 1 through 7. HI tests with these sera against ADV-3, 4, and 7 showed that the homologous serum produced HI titers ranging from 1:512 to 1:1024, while the heterologous serum produced a titer of slightly over 1:8. Thus, in contrast to the CF reaction(3), the HI test appeared to be type specific, at least in the cases of types 3, 4, and 7.

Attempts were made to follow the production of HA and CF activity from infected HeLa and MK cell sheets at different time intervals. Table V summarized the results of this work. Inoculum used was a seed that produced infective titers rapidly, although the correlative infectivities were not shown. In MK cells HA activity appeared after 4 hours, but CF activity was not found until 23 hours, indicating another difference between these factors. CF and HA activities from the infected HeLa cells were found only 72 hours after infection, which was different from the HA production in MK cells. HA production by ADV-3 was also studied in 2 other human

TABLE II. HA Titers of ADV-3, ADV-4, and ADV-7 after Storage under Various Conditions.

Virus	Source	Storage conditions	HA titer*
ADV-3	MK	2 years at 4°C	128
"	"	Conc. with PVP and stored 1 year at 4°C	>256
"	"	Inactivated 6 days by 1:4,000 HCHO at 37°C and stored at 4°C >1 yr	8
"	"	Conc. with PVP (10×) and stored 1 year at 4°C	>256
ADV-4	"	Conc. with 20% Methanol on Celite and stored >1 year at 4°C	>256
ADV-7	HeLa	Stored at –20°C for 1 year	64

* Using Grivet monkey cells.

TABLE III. Effect of Ultracentrifugation on Hemagglutinins of ADV-3.

Virus	Speed and time	Original titer (HA)	Original titer (CF)	Titer of supernatant (HA)	Titer of supernatant (CF)	Titer of resuspended pellet (HA)	Titer of resuspended pellet (CF)
HeLa	20,000 rpm/60 min.	2048		2048	—	128	—
"	20,000 rpm/90 min.	2048		1056	—	2048	—
MK	20,000 rpm/60 min.	8		16	—	4	—
"	40,000 rpm/90 min.	8		4	—	16	—
" with normal calf serum	39,000 rpm/90 min.	64		4	2	16	20
" without serum	39,000 rpm/90 min.	64		4	2	16	8 (AC)
MK 145	38,000 rpm/90 min.	16		0	—	32	—
MK	36,000 rpm/120 min.	512	3200	0	2000	320	40
"	36,000 rpm/180 min.	0	2000	0	1800	8	300

AC = anticomplimentary.

line cells (HB and Hep 2). HA titers of 1:32 to 1:64 were obtained from KB and Hep 2 cells 120 to 144 hours after infection with this virus.

Results summarized in Table VI showed that all of the HA activity was adsorbed by the grivet erythrocytes while most of the CF activity remained in the supernate and unadsorbed. An additional evidence of association was thus provided from soluble CF antigen common to all the adenoviruses. In certain experiments, low CF activity was found associated with red blood cell lysate. It was not determined whether this CF activity was identical with the soluble CF antigen.

Discussion. Our results confirm the original observation made by Rosen(1) regarding the capacity of adenoviruses, particularly ADV-3, to agglutinate erythrocytes obtained from grivet and rhesus monkeys. In our hands the grivet erythrocytes appeared to be more sensitive to agglutination by ADV-3 than those of the rhesus monkey. In contrast to Rosen's findings, these studies showed that ADV-4 and 7 also agglutinates the erythrocytes of these monkeys.

TABLE IV. Effect of Filtration on HA, CF, and TCID$_{50}$ Titers of Various Types of ADV.

Type	Pre-filtration HA	Pre-filtration CF	Pre-filtration TCID$_{50}$/ml*	Post-filtration HA	Post-filtration CF	Post-filtration TCID$_{50}$/ml*
3	8	7	5.9	8	<2	3.4
3	2	4	4.7	<2	<2	4.6
4	16	32	4.2	16	<2	3.6
4	32	8	4.5	32	2	3.9
4	16	16	4.1	>64	14	4.4
7	64	7	5.1	2	2	4.7
7	<2	<2	4.9	2	<2	4.6
7	<2	<2	4.4	2	<2	4.1

* Reciprocal of the dilution on Log_{10} showing 50% infective endpoint.

HA properties of ADV-3 were further studied. HA component of ADV-3 was separable from soluble CF antigen and from infectious virus. Centrifugation studies indicated that HA component may be larger than CF antigen but smaller than the infectious virus. HA activity of ADV-3 is separable from the infectious virus, as in vaccinia vi-

TABLE V. Production of HA and CF Antigens in Monkey Kidney and HeLa Cells Infected with ADV-3.

Hr after infection	MK CF	MK HA	HeLa CF*	HeLa HA*
0	<2	<2	<2	<2
4	<2	2	<2	<2
6	<2	8	<2	<2
12	<2	16	<2	2
24	2	32	<2	<2
48	—	256	<2	<2
72	—	—	5	512

* Avg titers of 6 cultures.

TABLE VI. Separation of HA from CF Activity of ADV-3 by Adsorption with Grivet Erythrocytes.

ADV-3	Titers CF	Titers HA
Original virus	1100	64
Supernate (post adsorption)	1120	<2
Erythrocyte lysate	216	—

ruses(7). On the other hand, filtration studies showed that HA component of adenovirus passed more readily through Seitz pads than CF antigen. Many factors might underline this discrepancy, *e.g.*, the difference in electrical charge on the particles or concentration of the different components.

HA activity was found earlier than CF antigen in fluids of MK cells infected with adenoviruses while the opposite usually obtains with viruses(8). This difference may reflect sensitivity of the tests or formation of each component at different times.

The increase in numbers of the adenovirus family has necessitated a search for a simple serologic test for identification. The value of CF test is rather limited, since it measures only a common soluble antigen, although modifications of this test by Pereira(5) and by Binn, *et al.*(6) have increased its usefulness. The specificity of the HI test with ADV-3, 4, and 7 Type 3 definitely indicates its value in diagnostic work. The HI test can be completed in less than 3 hours, thus it is relatively simpler than the CF test. Further, the reagents used in the HI are more stable than those used in the CF test.

Summary. Hemagglutinins of adenovirus Type 3, 4 and 7 were found to be separable from soluble CF antigen and infectious virus. Inhibition of HA activity with rabbit serum antibodies appeared to be type specific. The HA component was relatively stable to heat, formalin and storage at 4°C. It was more readily filterable than CF antigen and was produced earlier than the CF in infected MK cells.

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1. Rosen, L., *Virology*, 1958, v5, 574.
2. Prier, J. E., LeBeau, R. W., *J. Lab. & Clin. Med.*, 1958, v51, 495.
3. Rowe, W. P., Huebner, R. J., Bilmore, L. K., Parrot, R. H., Ward, T. G., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 570.
4. Osler, A. G., Strauss, J. H., Mayer, M. M., *Am. J. Syphilis, Gonorrhea & Venereal Dis.*, 1952, v36, 140.
5. Pereira, H. G., *J. Path. & Bact.*, 1956, v72, 105.
6. Binn, L. N., Hilleman, M. R., Rodriguez, J. E., Glabere, R. R., *J. Immunol.*, 1958, v80, 501.
7. Burnet, F. M., Boake, W. C., *ibid.*, 1946, v53, 1.
8. Henle, W., *Advances in Virus Research*, 1953, v1, 141.

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Lipid Metabolism in Cultured Cells. I. Factors Affecting Cholesterol Uptake.* (26524)

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The contribution of serum lipids to the nutrition of mammalian cells in culture has been described by Bailey *et al.*(1). It was shown that cells utilize triglycerides most rapidly, but also take up considerable quantities of cholesterol.

Rutstein *et al.*(2) reported that explants of human aorta accumulate cholesterol from a serum growth medium. It was claimed that cholesterol uptake was inhibited by addition of polyunsaturated fatty acids. A number of cell strains synthesize cholesterol *de novo* from simpler constituents in the medium(3). With an adequate supply of serum lipids, however, most of the cell cholesterol is derived from the serum(1). In view of the implication of cholesterol desposition in the etiology of atherosclerosis, it was of interest to determine the factors affecting cellular cholesterol uptake in an *in vitro* system.

Materials and methods. Preparation of growth media and propagation of the MB III strain of cells of mouse lymphoblasts has been described(1). Growth medium used for propagation of stock cultures was human

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placental cord serum diluted with one part of Gey's balanced salt solution (BSS). Adult human serum was prepared from the blood of normal individuals drawn after 12 hours of fasting. Rabbit serum was prepared from normal rabbit blood obtained by heart puncture from 12-hour fasted animals. In comparative studies on cholesterol uptake all sera were 'deactivated' by heating at 58° for 30 minutes to destroy a toxic factor found in most freshly prepared samples of human and rabbit serum. The heating did not change the cholesterol content of cells grown on the serum. Crystalline bovine albumin was obtained from Nutritional Biochemicals Inc., and highly purified samples of linoleic acid and stearic acid were supplied by the California Foundation for Biochemical Research. Cholesterol-4-C^{14} was obtained from Nuclear Chicago Inc. Radioactive samples were assayed using a halogen-quenched end-window Geiger-Muller tube in conjunction with the usual amplifier and scaler. Cholesterol was determined by the method of Pearson *et al.* (4) or where necessary by the more sensitive method of Zlatkis *et al.*(5). Determinations of cell population were made by counting suspensions in 0.2 × 0.625 Spiers-Levy hemocytometer chambers, and measurements of the dry weight of the cells were made by the colorimetric method of Bailey and Nejad(6). Free and esterified cholesterol were separated on small silicic acid columns as described by Wykoff(7).

Cholesterol emulsions for addition to tissue cultures were prepared by 2 methods as follows: (1) An alcoholic solution of cholesterol was dispensed rapidly with stirring into a sterile solution of distilled water containing 50 mg% Tween 80 (Atlas Powder Co.). Sterile balanced salt solution of double concentration was then added in equal volume. In this way stable emulsions of cholesterol microcrystals were obtained having particle sizes of from 0.5 to 1.5 μ when examined by dark-field microscopy. Clumping of the microcrystals resulted if the suspensions were autoclaved, or if the alcoholic solution was run directly into a Tween 80 solution in saline. Unstable emulsions also were obtained if cholesterol concentration exceeded 100 mg% in the final solution. (2) Finely powdered cholesterol was shaken overnight in a sterile solution of crystalline albumin (2%) in isotonic saline. Excess cholesterol was then removed by high speed centrifugation. Use of these solutions was not practicable in those experiments involving alterations in serum protein:cholesterol ratios.

For studies on cholesterol uptake, cells from 3- or 4-day-old stock cultures were harvested by centrifugation, washed in balanced saline solution and resuspended in saline. Portions of the suspensions containing approximately one million cells were dispensed into roller tube cultures and the test medium was added in a final volume of 1 ml. After 4 days growth, cells were harvested by centrifugation and washed with 1 ml of saline to remove traces of medium. Cholesterol was extracted by heating at 45° for 1 hour with 3 ml of 1:1 ethanol-ether mixture. Extracted cells were centrifuged off, the extract was decanted and cells were air dried for 1 hour in an oven at 110°. Dry weight was determined colorimetrically as described previously. The ethanol-ether extract was evaporated to dryness and the residue was extracted twice with boiling petroleum ether. Where necessary, an aliquot of the petroleum ether was used for measurement of radioactivity, and the remainder was then evaporated to dryness for determination of cholesterol. Cholesterol content of serum samples used in the growth medium was determined by using a similar double solvent extraction method.

Results. Variation of serum type in medium. MB III cells were grown on adult human serum (11 samples), human placental cord serum (8 samples) and adult rabbit serum (6 samples). Each serum was used at a concentration of 50% in the medium and was diluted with an equal volume of balanced saline. Cells were harvested after 4 days. Growth was more rapid in cord serum (mean population increase 5.7 fold) than in adult serum (mean increase 3.5 fold). Cells grown on placental cord serum had a mean cholesterol content of only 2.25% and mean serum cholesterol was 97 mg% (Table I). Cells

TABLE I. Cholesterol Content of MB III Cells Grown on Different Serum Samples.

Type of serum	No. of samples	Serum cholesterol (mg %)	Cell cholesterol (%)
Human adult	11	218.7 ± 49.6*	3.74 ± .62*
" cord	8	97.4 ± 35.3	2.25 ± .23
Adult rabbit	6	85.8 ± 29.5	7.80 ± 2.29

* Mean and stand. dev.

Cells were grown for 4 days on the different serum samples diluted with one part of balanced saline. Cells were harvested and cholesterol content was determined as described in *Methods* section.

grown on human adult serum had a higher cholesterol content of 3.74%, and mean serum cholesterol was 219 mg%. Cells grown on rabbit serum had the highest cholesterol content, mean 7.8% although rabbit serum itself had the lowest cholesterol level (mean 86 mg%).

Variation of concentration of serum in medium. Concentration of cord serum in the medium was varied from 20% to 100%. The cholesterol level was thus varied between 16 and 80 mg% without altering the ratio of cholesterol to other serum components. Cells grew more slowly at the lower serum concentrations. There was no significant change in cholesterol content of cells despite the 5-fold range of cholesterol concentrations in which they were grown (Table II).

Addition of free cholesterol to medium. The medium, consisting of 50% human placental cord serum and balanced salt solution, was supplemented with relatively small amounts of free cholesterol in the form of an emulsion with Tween 80. The medium contained initially 40 mg% cholesterol provided by the serum, and the emulsion was added to the cell cultures in the concentrations shown (Fig. 1). The highest concentration (56 mg%) represented an additional 16 mg % over that normally provided by the serum (Fig. 1). At the end of experiment cells were harvested by low speed centrifugation to prevent sedimentation or flotation of cholesterol. Sedimented cells were resuspended twice in balanced saline and recentrifuged to remove any particulate matter adhering to the exterior of the cell membrane. Microscopic examination of the cells using phase optics, and also staining with Sudan black, confirmed the presence of small cholesterol microcrystals within the cell. The cells were analyzed for cholesterol in the usual manner. Addition of relatively small amounts of excess cholesterol to the medium brought about a striking increase in cholesterol content of cells (Fig. 1). The stability of the single microcrystals in the emulsion was good up to levels of 10 mg%, but above this level, clumping of the microcrystals was brought about following a few hours incubation in the serum medium.

TABLE II. Variation in Cell Cholesterol by Varying Serum Cholesterol in the Medium.

Serum cholesterol (mg %)	16	24	32	40	60	80
Cell cholesterol (%)	2.2	2.3	2.5	2.3	2.2	1.9

Cells were grown in a medium consisting of balanced saline supplemented with human placental cord serum having 80 mg % cholesterol. Percentage of serum added was varied to give cholesterol concentrations shown.

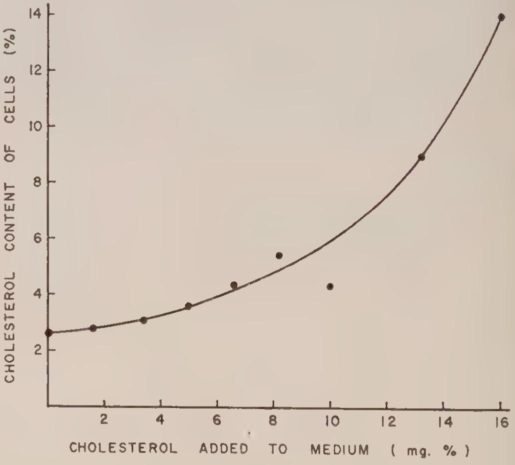

FIG. 1.

It seemed reasonable to assume from the results in Fig. 1 that the added cholesterol was taken up preferentially. To test this, cells were grown in a medium of similar composition, but with an added emulsion of cholesterol 4-C^{14}. By measuring the specific activity of the cellular cholesterol it was possible to calculate the percentage derived from the serum cholesterol and the percentage derived from the added cholesterol. It was found (Table III) that the added cholesterol

TABLE III. Distribution of Serum Cholesterol and Cholesterol-C^{14} in Cells.

C^{14} cholesterol added (mg %)	Total cholesterol in medium (mg %)	Cell cholesterol (%)			
		Total	C^{14}	Unlabelled	Additional unlabelled
0	70	2.0	0	2.0	0
2.5	72.5	2.0	.3	1.7	-.3
5.0	75	4.3	1.3	3.0	1.0
10.0	80	4.6	1.9	2.8	.8
15.0	85	5.9	2.8	3.1	1.1

Cord serum medium was supplemented with increasing amounts of cholesterol-C^{14} emulsion giving final concentrations shown. Specific activity of cholesterol in the harvested cells was measured. In this way the fraction derived from the serum cholesterol (4th and 5th columns) was determined. "Additional unlabelled" cholesterol is calculated as the difference between cholesterol content of cells grown on serum cholesterol alone (2.0%) and the contribution of the serum cholesterol in cells grown on a medium supplemented with labelled C^{14} cholesterol. Addition of unbound C^{14} cholesterol to medium resulted in increased uptake of unlabelled serum cholesterol.

was taken up preferentially compared with its relative concentration in the medium. This, however, did not account completely for the increase in the cells as is shown by the last column in Table III. Addition of free cholesterol also resulted in an increase in uptake of serum cholesterol as compared to uptake from this fraction in control cultures.

Because of the preferential uptake of unbound cholesterol, it was of interest to determine the pattern of uptake when cholesterol was added in a protein-bound form. Cells were grown in 50% cord serum medium at a total cholesterol concentration of 86 mg%. 70 mg% of this was provided by the serum and 16 mg% by adding an equal volume of a solution of cholesterol C^{14} in 2% crystalline bovine albumin (*see* Methods section). After 4 days growth the cells contained 1.3% cholesterol, of which only 14% was derived from labelled albumin-bound cholesterol. Since the albumin-bound fraction provided 18% of the cholesterol in the medium, it was concluded that cholesterol added in combination with protein (in contrast to unbound cholesterol) was taken up at approximately the same rate as serum cholesterol.

Variation of albumin content of medium. Four samples of serum from normal human adults having low serum cholesterol levels, and 4 samples of serum from atherosclerotic patients were pooled. Cells were grown in both samples of pooled serum at a level of 50% in the medium and cultures were supplemented with increasing concentrations of crystalline bovine albumin (Table IV). Mean cholesterol content of cells grown on the normal serum was 3.2% compared with 4.1% for cells grown on the atherosclerotic serum. No significant change was produced in the values over the range of albumin concentrations studied (Table IV).

Addition of fatty acids. Three groups of cultures were set up in 50% human placental cord serum. Cholesterol emulsion was added, giving concentrations in the final medium of 0, 1, 2, 3, 4, 5 and 6 mg% of added cholesterol. One group of cultures was retained as controls, and the other 2 groups were supplemented with linoleic acid or stearic acid emulsion (0.5 mg%). The cells were harvested after 4 days of growth. There was no significant difference in cholesterol content of the 3 groups (Control 5.4%; linoleic acid supplemented 4.7% and stearic acid supplemented 5.1%). A 3-fold increase in fatty acid concentrations (1.5 mg%) with a different sample of serum gave a similar result

TABLE IV. Effect on Cell Cholesterol of Increasing Concentrations of Albumin in the Medium.

Albumin added (mg %)	0	100	300	1000	2000
Cell cholesterol (%)					
Pooled normal serum	3.2	3.1	3.0	3.0	2.7
" athero. "	4.1	4.2	4.0	4.5	4.5

Four samples of normal human adult serum having low cholesterol levels were pooled. Mean serum cholesterol was 146 mg %. Four serum samples from atherosclerotic patients having a mean serum cholesterol of 233 mg % were also pooled. Cells were grown in samples of both pooled sera diluted with one part of balanced saline solution and supplemented with crystalline bovine albumin at concentrations shown.

(Control 4.2%; linoleic acid supplemented 3.9% and stearic acid supplemented 4.3%). Higher concentrations of free fatty acids than those used here were toxic to the cells.

Relative importance of free and esterified cholesterol. About two-thirds of the cholesterol in serum is normally present in the esterified form. Experiments were performed to determine the relative contributions of these 2 fractions to the cell cholesterol. It was found that the cell cholesterol was almost exclusively (95-100%) in the unesterified form, but that, paradoxically, most of the decrease in the medium occurred in the ester fraction. Supplementing the medium with free cholesterol-C^{14}, however, showed that about half of the cell cholesterol was derived from the free cholesterol of serum. Following incubation for the 4-day growth period, about 20% of the label in the medium was in the ester fraction. The labelled ester cholesterol found in the medium results from esterification exchange catalyzed by the serum. It seems probable also that the cells contain a cholesterol esterase which breaks down the esters to free cholesterol following absorption.

Discussion. It has been shown that uptake of serum cholesterol by cells in culture is related to cell type(1). In a series of cells grown in human placental cord serum, cholesterol content varied from a high of 2.8% for the FL strain of human amnion to as low as 0.6% for a strain of rat adrenal cells. The experiments reported here demonstrate that with a given cell type, cell cholesterol is also dependent upon the type of serum used in the growth medium. It is also apparent that cell cholesterol is determined by factors other than (or in addition to) serum cholesterol level as is seen by comparing cholesterol content of cells grown on rabbit serum with that of cells grown on human placental cord serum (Table I). Cholesterol level in rabbit serum was less than in cord serum. Cells grown on rabbit serum, however, had over 3 times the cholesterol content of those grown on cord serum.

The results (Table II) indicate that the important parameter in determining cell cholesterol levels is the ratio of cholesterol to other components of the serum. This was confirmed in experiments (Fig. 1) where unbound cholesterol was added to the medium in the form of an emulsion. Relatively small increments in unbound cholesterol brought about large increases in cell cholesterol. When the same concentrations of cholesterol were added in the form of a protein complex with crystalline albumin, however, there was no corresponding effect on cell levels. This suggests that one determining factor may be the relative ratio of protein-bound to unbound cholesterol in serum. That this is not the only factor, however, is shown by the fact that increasing the albumin content of the medium up to 80% above normal levels did not alter cholesterol content of the harvested cells (Table IV). Since most of the cholesterol in serum is bound to an alpha-globulin fraction,[†] it is possible that cholesterol binding by proteins other than albumin is more important. It would be expected that both qualitative as well as quantitative parameters should be considered in evaluating the importance of protein-bound cholesterol. Thus for example, when serum is shaken with free cholesterol-C^{14}, most of the bound radioactivity appears in the alpha-globulins[†] and relatively little in the albumin fractions. When albumin is shaken alone with cholesterol-C^{14}, however, it binds considerable amounts of radioactivity.

The variation in uptake of cholesterol from different serum samples was not due to differences in free fatty acid content of the serum. Concentration of cellular cholesterol was not affected by addition of linoleic acid or stearic acid to the medium in concentrations of up to 1.5 mg%. We have thus failed to observe in this cell system the effects on cholesterol uptake which were noted by Rustein *et al.*(2) in explants of aorta tissue when similar concentrations of free fatty acids were used.

The question of the relative contribution of free cholesterol and cholesterol esters to the cellular cholesterol is not clear from the results. In serum medium alone, only cholesterol esters are depleted, whereas it is apparent from the experiment using labelled cholesterol that both free and ester are taken up. The most probable explanation of the results is that both free and ester cholesterol are taken up by cells. Cholesterol esters are then hydrolyzed by the cells and are deposited as free cholesterol. Any excess is then excreted back into the medium as free cholesterol. We have observed the functioning of this excretion process by using cells grown on C^{14} labelled cholesterol and later transferred to unlabelled medium(8). The role of the serum proteins in this excretion process is being investigated.

[†] As determined by chromatography on DEAE ion-exchange cellulose columns. (J. M. Bailey and Edward Walsh, unpublished data).

Summary. 1. Some of the factors influencing uptake of cholesterol by a strain of mammalian cells growing *in vitro* have been examined. 2. Cholesterol uptake by cells is dependent upon the type of serum used in the medium. Cells grown on rabbit serum had a higher cholesterol content than cells grown on human adult serum or human placental cord serum. Rabbit serum, however, had the lowest serum cholesterol level. 3. Cholesterol content of cells was not influenced by concentration of serum cholesterol in the growth medium provided the relative proportions of cholesterol and serum protein were not changed. 4. When emulsions of free (non-protein bound) cholesterol were added to serum medium, relatively small increases in cholesterol content of the medium resulted in large increases in cholesterol content of the cells. 5. By use of C^{14} labelled cholesterol, it was shown that unbound cholesterol was taken up preferentially as compared with cholesterol in the protein-bound form. 6. Cholesterol content of cells was not influenced by addition of stearic acid or linoleic acid to the growth medium. 7. Cells utilized both free and esterified serum cholesterol. The ester was apparently hydrolyzed intracellularly since the cell cholesterol was almost entirely in the unesterified form. 8. It is concluded that the main factor controlling cellular cholesterol uptake may be the relationship between serum cholesterol and the binding power of the serum proteins, rather than the cholesterol level itself.

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1. Bailey, J. Martyn, Gey, George O., Gey, Margaret K., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 686.

2. Rutstein, D. D., Ingenito, E. F., Craig, J. M., Martinelli, M., *Lancet,* 1958, 545.

3. Azarnoff, D. L., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 680.

4. Pearson, S., Stern, S., McGavack, R., *Anal. Chem.,* 1953, v25, 813.

5. Zlatkis, A., Zak, B., Boyle, A., *J. Lab. Clin. Med.,* 1953, v41, 486.

6. Bailey, J. Martyn, Maymandi-Nejad, A., *ibid.,* 1961, in press.

7. Wykoff, H. D., Parsons, J., *Science,* 1957, v25, 347.

8. Bailey, J. Martyn, Gey, George O., *Fed. Proc.,* 1959, v18, 17.

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Interaction of Compound 48/80 and Stilbamidine with Strandin.* (26525)

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Histamine release from mast cells may be effected by a variety of substances, among which are chlorpromazine(1), protamine(2), the thiazine dye, toluidine blue O(2), compound 48/80(2) and stilbamidine(2,3). All of these compounds interact markedly with heparin and chondroitin sulfate. MacIntosh, reviewing the behavior of a number of histamine releasers, has found a parallel between histamine release and heparin combining power(2).

Recent work in this laboratory has indi-

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* Supported by a grant from National Tay Sachs' Assn.

cated that chlorpromazine(4), protamine(5) and toluidine blue O(6) all interact with the brain ganglioside strandin, to produce respectively; a soluble product, turbidity leading to precipitation, and metachromasy. In view of these facts we wished to ascertain whether compound 48/80 and/or stilbamidine would also interact with strandin.

Materials and methods. 1. The brain ganglioside, strandin, was obtained from the cortical gray matter of a case of Tay Sachs' disease by the method of Folch, Lees and Sloane-Stanley(7). Tissue was maintained at –8°F in the deep freeze prior to extraction. Strandin was analyzed by the neuraminic acid method of Saifer and Gerstenfeld(8), assuming an average value of 30.5 mg of neuraminic acid per 100 mg of strandin(9). 2. Materials were obtained as follows: Compound 48/80 from Burroughs Wellcome and Co. Inc.† 3. Stilbamidine (4,4′-stilbene dicarboxamidine) di (β-hydroxyethane sulfonate), from Delta Chemical Works, N. Y. 4. Chondroitin sulfate (CSA) from Nutritional Biochemicals Corp., Cleveland, Ohio. 5. Germanin(3,3′-ureidobis (8 - (benzamido-p-toluido)-1,2,5-napthalene trisulfonic acid)) from Delta Chemical Works, Inc., N. Y. 6. Lysozyme (2× crystallized) from Worthington Biochemical Corp., Freehold, N. J. 7. Protamine Sulfate (Salmine) C.P., from Mann Research Laboratories, N. Y. 8. Poly-l-lysine from Schwarz BioResearch, Inc., Mount Vernon, N. Y. 9. Toluidine Blue O (TBO) (Total dye content of 94%) from National Aniline Division, Allied Chemical and Dye Corp., N. Y. 10. Acriflavine, N.F. (2:8 diamino-N-methyl acridinium chloride) from Abbott Laboratories, North Chicago, Ill. 11. All other chemicals used were of reagent grade. 12. Measurements of turbidity were made using the Bausch and Lomb Spectronic-20 Spectrophotometer set at a wavelength of 420 mμ. Metachromasy was measured with the same instrument, using as index of its intensity the Optical Density Ratio (O.D.R.) which is equal to (O.D. 550/O.D. 635)(6). 13. Ultraviolet absorption spectra were measured using the Beckman DU Spectrophotometer. 14. Fluorescence measurements were made with the Coleman Electronic Photofluorometer Model 120. For excitation the optical filter 12-221 (B_1) was used. For transmission of the fluorescent light of acriflavine, the yellow optical filter 14-212 (PC-2) was used. For transmission of the fluorescence of stilbamidine, the blue optical filter 12-223 (B3) was used. The molecular weight of strandin in water solution is not known with certainty(10). Compound 48/80 is likewise a material of uncertain molecular weight (11). Hence in dealing with these compounds we were unable to use molarity as a measure of concentration, and were forced to utilize such expressions as mg/L or mg%. In the case of stilbamidine isethionate (M.W. = 516.59) we have employed the millimolar expression for concentration in conjunction with the metric concentration, since the latter is required for purposes of comparison with compound 48/80.

Results. A) *Direct interaction of strandin with histamine liberators.* As indicated in Fig. 1A, the turbidimetric titration of 3 ml of 0.37% strandin with 1% of compound 48/80 at 6°C results in a marked increase in optical density at 420 mμ. The increase becomes maximal upon addition of 20 mg/L of the substance. The increase is of the order of 0.610 optical density units, and further addition of compound 48/80 resulted in a reduction in turbidity. The pH change was from 5.7 to 3.5. To ascertain whether this change in pH was significant, 3 ml of 0.37% strandin were titrated with 0.1N HCl down to pH 3.5 at 6°C. There was no change in turbidity. In the case of stilbamidine (Fig. 1B), the interaction was of less magnitude. A maximal turbidity increase of 0.420 optical density unit occurs on addition of 50 mg/L (0.097mM/L) of 1% stilbamidine to 3 ml of 0.37% strandin, followed by a slight decline on addition of further amounts of stilbamidine. The pH change which accompanies the titration is from 5.7 to 5.0 units.

Further evidence of direct interaction was obtained by studying the effect of strandin on

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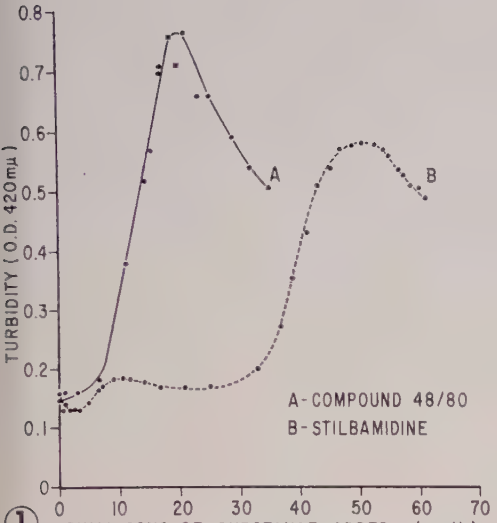
† We wish to thank Dr. W. P. Colvin, Med. Dept., Burroughs Wellcome and Co., for providing a supply of this compound.



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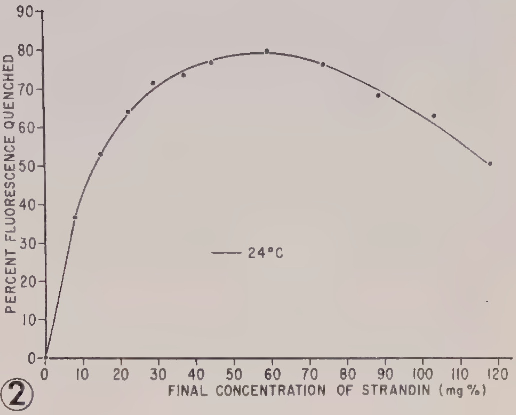

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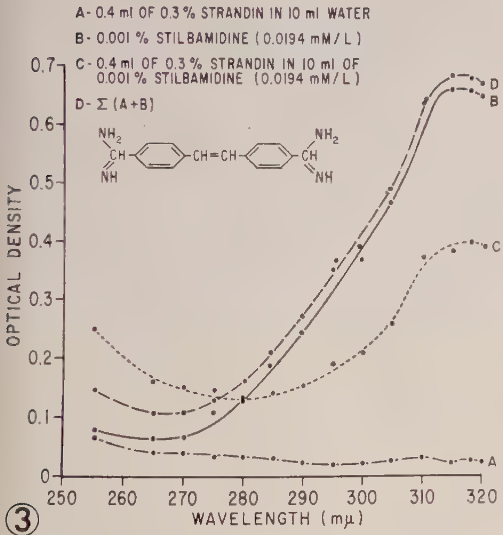

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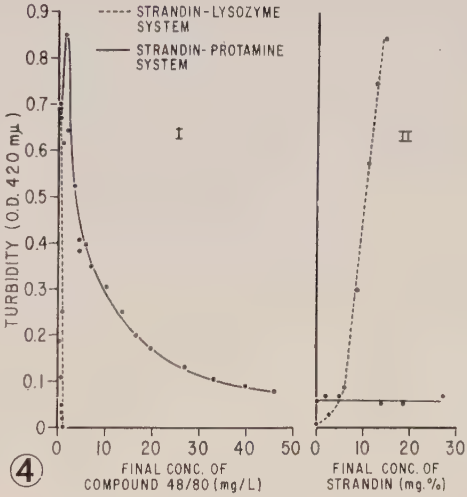

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FIG. 1. Turbidimetric titration of strandin with stilbamidine and compound 48/80 at 6°C. 3 ml of 0.37% strandin titrated with indicated amounts of stilbamidine (1.0%) (19.4 mM/l) and compound 48/80 (1.0%). pH at start was 5.7; at end of stilbamidine titration, 5.0 and at end of compound 48/80 titration, 3.5.

FIG. 2. Fluorescence quenching of stilbamidine by strandin. 5 ml total volume containing 10 mg % of stilbamidine (0.194 mM/l) plus indicated amounts of strandin.

FIG. 3. Effect of strandin on UV absorption spectrum of stilbamidine. Concentrations as indicated in legend. Initial pH of stilbamidine solution was 6.8; after addition of strandin, 6.1.

FIG. 4. Repression of strandin-protein turbidity by compound 48/80 and reversal of lysozyme system by strandin. 3 ml total volume in each case, containing 0.1 ml of 1% protamine sulfate, and 0.3 ml of 0.3% strandin, plus indicated amounts of compound 48/80 and 0.1 ml of 1% lysozyme plus 0.1 ml of 0.3% strandin plus indicated amounts of compound 48/80. pH change for the protamine system was from 3.8 to 3.3; for the lysozyme system, from 6.8 to 6.2.

the fluorescence of stilbamidine. The fluorescence of stilbamidine (10 mg%) (0.194mM/L) is quenched by addition of varying amounts of a 0.74% strandin solution (Fig. 2). The effect is a function of the strandin concentration since, with increasing concentration of strandin, the effect rises to a maximum and then decreases. Initial pH of the stilbamidine solution was 7.0. Upon addition of 118 mg% of strandin at 24°C, pH dropped to 6.8. While compound 48/80 does not exhibit fluorescence, it does possess the capacity to reverse the quenching of fluorescence of acriflavine by strandin(6).

Strandin causes a marked alteration in the UV spectrum of stilbamidine (Fig. 3C). The theoretical curve resulting from the algebraic addition of absorption of strandin (0.4 ml of 0.30% in 10 ml of water) and of 0.001% (0.0194 mM/L) of stilbamidine is shown in curve 3D. This curve shows a slightly greater absorption than that of stilbamidine alone (curve 3B). The difference to be stressed, however, is that between curves 3D and 3C, since the latter represents the observed effect of strandin (0.4 ml of 0.30%) on 10 ml of 0.001% stilbamidine. A rise occurs in optical absorption in the region below 280 mμ and a profound drop between 280 mμ and 320 mμ, giving further indication of interaction between the 2 compounds.

We were unable to demonstrate a significant effect of strandin on the UV spectrum of compound 48/80.

B) *Indirect evidence of interaction with strandin.* 1. *Interference with Protein-Strandin interaction.* The interaction between strandin and the basic proteins, lysozyme and protamine, has been demonstrated(5). A number of physicochemical factors which influence this interaction have also been described. We have found that both the protamine-strandin turbidimetric interaction product and the lysozyme-strandin product are sensitive to the action of compound 48/80 (Fig. 4). The lysozyme system is the more sensitive, and within the concentration limits studied, it is clearly reversed by readdition of strandin. The pH change for the lysozyme-strandin system, titrated with compound 48/80 was from 6.8 to 6.2; on retitration with strandin, pH dropped to 6.1 indicating that lowering the pH was not the cause of the preceding effect. Reversal was not found to be the case with the protamine system which had been suppressed by 48/80. In the case of the protamine system there is an initial rise in turbidity upon addition of the first aliquot of 48/80. A phenomenon similar to this was observed in the interaction of cetyltrimethylammonium bromide with the protamine-strandin system(12).

In dealing with stilbamidine we found that results similar to those shown in Fig. 4 were obtained with both lysozyme and the synthetic polypeptide, poly-l-lysine. The situation with protamine was entirely different. We found here that addition of stilbamidine caused a rise in turbidity to a maximum, followed by a slight fall. Readdition of strandin caused a rise in turbidity to an even greater value than did addition of stilbamidine alone.

2. *Interference with toluidine blue O-strandin metachromasy.* Titration of the strandin-toluidine blue O (TBO) system (a 20 ml solution containing 1.06×10^{-5}M TBO and 0.45 ml of strandin (.30%)) with 0.5% stilbamidine (9.68 mM/L) causes a pronounced reduction in metachromasy, as measured by optical density ratio (O.D.R.) so that it has dropped from a value of 1.17 to 0.47 upon addition of 0.04 ml of stilbamidine. Upon adding 0.2 ml of stilbamidine solution, O.D.R. reaches a value of 0.40 unit and the corresponding pH change is from 7.2 to 6.3. Attempts to reverse this phenomenon by further addition of strandin were not successful.

Somewhat similar results were obtained on titration of the strandin-TBO system with 0.5% compound 48/80, except that upon addition of the first few aliquots of compound 48/80, a slight rise in metachromasy occurred, followed thereafter by a profound fall. This system could not be reversed by readdition of strandin. The pH change was from 6.0 to 6.3.

3. *Effect of strandin on polysulfonate-stilbamidine interactions.* Mota *et al.*(13) have indicated that addition of compound 48/80 or of stilbamidine to a solution of heparin, li-

quoid or cellulose sulfate will result in precipitate formation. We have found that 48/80 and stilbamidine also precipitate chondroitin sulfate (CSA) and Germanin. The latter compound possesses 2 features in common with heparin; it inhibits clotting of blood and has pronounced metachromatic properties (14).

We found that in the case of compound 48/80, strandin caused only minor reduction in turbidity of either the heparin, the CSA or the Germanin systems. A change of about 49% occurred when a stilbamidine-Germanin system (containing 1.19 millimoles/L of Germanin and 1.13 millimoles/L of stilbamidine in a total volume of 3 ml) was titrated with 1.0 ml of 1.0% strandin. Allowance was made, in calculation, for the dilution effect, and pH was found to drop from 6.1 to 5.0. For this system it was found that lowering the pH by titration with 0.1N HCl caused an *increase* in turbidity which at pH of 5.0 was not very marked. It was therefore concluded that the pH change attendant on this reaction was not significant. In the case of a CSA-stilbamidine system (containing 0.17% of CSA and 0.48 millimoles/L of stilbamidine in a total volume of 3 ml), the reduction in turbidity attendant on addition of 1 ml of 1% strandin was also 49%. Allowance was made for the dilution effect of the addendum, and the pH change was from 6.1 to 5.7. Titration of this system with 0.1N HCl revealed that until a pH of 3.3 had been reached, no change in turbidity occurred. Upon reaching pH 3.3, a rise in turbidity with time occurs. Thus the pH change is again of little consequence.

Discussion. We have demonstrated that some substances (which are also histamine liberators) such as compound 48/80 and stilbamidine, interact with the brain ganglioside, strandin. From a chemical point of view the reaction falls into the general class of reactions determined by the anionic character of strandin, giving rise to a series of phenomena which are also characteristic of the sulfated mucopolysaccharides. The interaction of these substances with strandin probably proceeds *via* the carboxyl groups of the neuraminic acid moiety of strandin, the secondary nitrogen atom of the compound 48/80 polymeric chain, and the guanidine groups of the stilbamidine molecule.

In view of the evidence indicating direct interaction between the cationic compounds (CC) and strandin (S), we may postulate the following reversible equilibrium (disregarding charge):

$$CC + S \rightleftarrows (CC\text{–}S) \quad \ldots\ldots\ldots\ldots \quad 1$$

Evidence supporting this picture of the reaction comes from the turbidimetric studies at 6°C, in the case of both compound 48/80 and of stilbamidine; from quenching of fluorescence of stilbamidine by strandin, and from the effect of strandin on the UV-spectrum of stilbamidine. The tendency of the interaction product to dissociate, indicating weak bonding (which is in turn designated by the relative size of the arrows in the above equation), is substantiated by the weak retention of such cationic substances by strandin during an equilibrium dialysis experiment we have performed.

The effect of strandin on interaction of stilbamidine with Germanin and CSA, and the interference of both compound 48/80 and stilbamidine with strandin-TBO metachromasy and with strandin-protein interaction may be interpreted in terms of competitive equilibria. This interpretation is not, however, applicable to the interaction of stilbamidine with the protamine-strandin system, in which case an increase in turbidity results which may be due to a common guanidine group being present in both stilbamidine and in the arginine of the protamine molecule.

These data show that some cationic substances which are known histamine liberators not only interact with heparin as reported by MacIntosh(2), but also with the brain ganglioside, strandin. Whether all cationic histamine liberators will also interact with strandin, requires further investigation.

Summary. 1. Both compound 48/80 and stilbamidine interact directly with strandin at 6°C to form turbidimetric interaction products. 2. The fluorescence of stilbamidine is quenched by strandin, the effect being a func-

tion of the strandin concentration. 3. Strandin exerts a marked effect upon the UV spectrum of stilbamidine, optical density rising above the theoretical below 280 mμ, and falling well below the theoretical curve above 280 mμ. 4. Compound 48/80 causes a reduction in turbidity of the strandin-lysozyme and the strandin-protamine systems. In the case of the lysozyme system the reaction was markedly reversed by readdition of strandin. This was not the case with the protamine system. With stilbamidine similar results were obtained with both lysozyme-strandin and poly-l-lysine-strandin. In the case of protamine-strandin, stilbamidine caused an increase in turbidity of the system. 5. Both compound 48/80 and stilbamidine suppress the metachromasy of the toluidine blue O-strandin system. 6. Strandin was found effectively to suppress the interaction between chondroitin sulfate and stilbamidine, and the interaction between Germanin and stilbamidine. 7. A mechanism involving a reversible equilibrium between these compounds and strandin has been proposed to explain the results obtained.

1. LeBlanc, J., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 238.

2. MacIntosh, F. C., Histamine and Intracellular Particles, in Ciba Foundation Symposium on Histamine, edited by Wolstenholme, G.E.W. and O'Conner, C. M., Little Brown and Co., Boston, 1956, pp. 20-35.

3. Riley, J. F., The Mast Cells, E. and S. Livingstone Ltd., Edinburgh and London, 1959, pp. 59-63.

4. Harris, A. F., Saifer, A., Volk, B. W., PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 542.

5. Harris, A. F., Saifer, A., *J. Neurochem.*, 1960, v5, 383.

6. ———, *ibid.*, 1960, v5, 218.

7. Folch, J., Lees, M., Sloane-Stanley, G. H., *J. Biol Chem.*, 1957, v226, 497.

8. Saifer, A., Gerstenfeld, S., *J. Lab. Clin. Med.*, 1957, v50, 17.

9. Folch, J., Lees, M., *Amer. J. Dis. Child.*, 1959, v97, 730.

10. Klenk, E., Neuraminic Acid in Ciba Foundation Symposium on the Chemistry and Biology of the Mucopolysaccharides, Little Brown and Co., Boston, 1958, p. 305.

11. Baltzly, R., Buck, J. S., deBeer, E. J., Webb, F. J., *J. Am. Chem. Soc.*, 1949, v71, 1301.

12. Harris, A. F., Saifer, A. Unpublished observations.

13. Mota, I., Beraldo, W. T., Junqueira, L. C. U., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 455.

14. Harris, A. F., Sobel, A. E., *Arch. Biochem. Biophysics*, 1958, v74, 345.

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Fat Excretion in Dogs Lacking Both Bile and Pancreatic Juice.* (26526)

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When dogs are deprived of bile or pancreatic juice they excrete 40 to 60% of ordinary dietary triglycerides over a wide range of intakes(1). However, degree of steatorrhea has not been measured in dogs when both bile and pancreatic deficiency occur simultaneously. This investigation was undertaken to make this determination.

Methods. Experiments were performed on normal dogs and on dogs lacking pancreatic juice, bile or both. Surgical procedures were performed aseptically on mongrel females weighing 9 to 18 kg with due regard for the requirements of proper animal care. Pancreatic deficiency was produced by removing all of the pancreas except the uncinate process(2). Subsequent necropsy and histological study showed no connection between duodenum and pancreatic remnant. None of the dogs became diabetic. External bile fistulas were prepared by a modified Rous-McMaster technic(3). The combined deficiency was produced by simultaneous pancreatectomy and preparation of the bile fistula. A return

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* Supported by grant from Nat. Inst. of Arthritis and Met. Dis.

TABLE I. Fecal Fat Excretion in 4 Normal Dogs.

Food intake/day	Fecal fat, g
Control diet*	.7 ± .2‡
plus 25 g oleic acid	1.0 ± .2
" 50 g " "	4.1 ± 1.1
" 75 g " "	10.1 ± 2.9
" 50 g olive oil	1.9 ± .7
" 75 g " "†	2.0 ± .1

* 10 g fat.
† 2 dogs only on this test.
‡ Mean ± S.D.

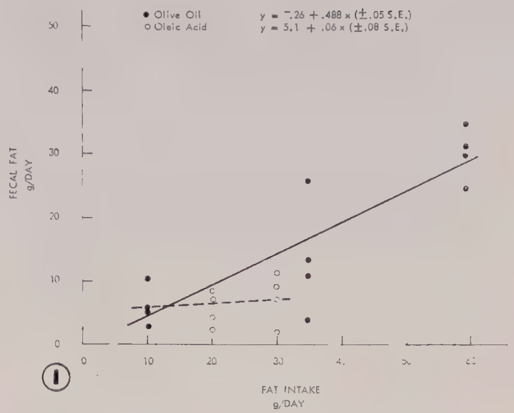
cannula of Tygon tubing was implanted in the duodenum in all groups for administration of saline(3). A many-tailed binder covered the cannulas.

Test meals, fed once daily starting approximately one week after operation, were completely consumed. They consisted of a control meal of 300 g of canned dog food ("Friskies", Carnation Co.) supplemented with sucrose (50 g), casein hydrolysate (25 g) and brewers yeast (2 g); or the control meal to which were added varying amounts of olive oil or oleic acid. The order of feeding was randomized to control the possible effects that time after operation and diet schedule might have on fat excretion. Feces were collected and refrigerated for the last 5 days of 7 day test periods. Fecal fat excretion was then estimated on duplicate aliquots of the total blended fecal sample as the petroleum ether soluble material extracted in 7 hours(4).

Results. Normal dogs (Table I). Fecal fat excretion did not increase significantly when 25 g of oleic acid or olive oil were added to the control meal, indicating that complete absorption occurred. In confirmation of an earlier study(5) 50 g and 75 g of oleic acid were incompletely absorbed since fecal fat excretion was 6.8% and 12.5% of the intakes respectively

$$\left[\begin{array}{c}\%\ \text{fat excretion} = \\ \dfrac{\text{Total fecal fat} - \text{control diet fecal fat}}{\text{Total dietary fat}} \times 100\end{array}\right].$$

Accordingly, to minimize a possible laxative effect of these larger doses, operated animals were fed only 10 g and 20 g of oleic acid.



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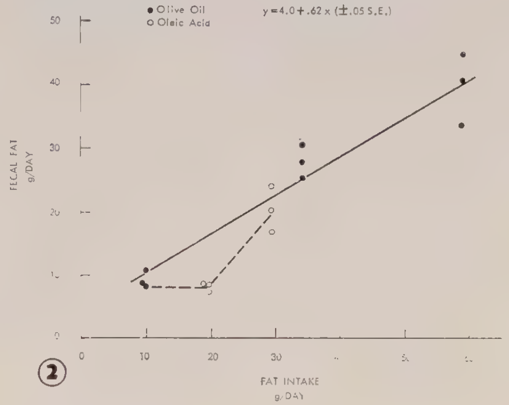

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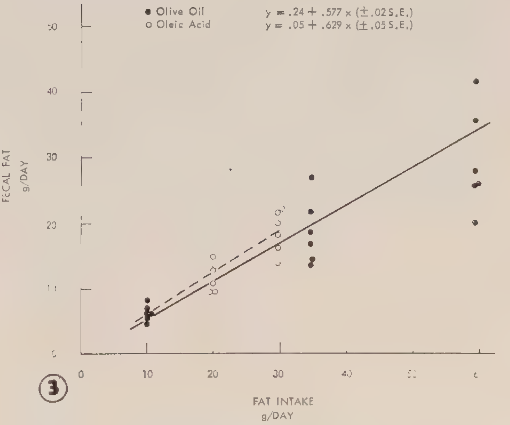

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FIG. 1. Absence of pancreatic juice.
FIG. 2. Absence of bile.
FIG. 3. Absence of bile and pancreatic juice.

Pancreatic deficiency (Fig. 1). As in an earlier investigation(5), the dietary triglyceride was poorly assimilated. The calculated regression line on 4 dogs indicates an assimilative deficiency of nearly 50%. Fecal fat did not increase significantly when 10 g and 20 g of oleic acid were added to the control meal, comparable to the finding when 25 g were fed to normal dogs.

Bile deficiency (Fig. 2). In 3 dogs fecal fat excretion was 62% of the intake of olive oil, comparable to the defect for triglyceride observed by others in bile deficiency(1). Fat excretion was unchanged when 10 g of oleic acid were added to the control meal. However, when 20 g were fed, the deficiency in absorption was comparable to that for olive oil.

Combined pancreatic-bile deficiency (Fig. 3). Assimilative deficiencies for olive oil and oleic acid were nearly 58% and 63% respectively when 6 dogs were deprived of both bile and pancreatic juice.

Discussion. Approximately 40% of the dietary triglyceride and fatty acid were assimilated even when neither bile nor pancreatic juice was present. This finding fails to support an implication from a study in rats (6) that triglyceride and fatty acid assimilation are virtually absent in combined pancreatic-bile deficiency. When corn oil, corn oil fatty acids or oleic acid were placed in the duodenum of rats from which both pancreatic juice and bile were excluded, no corn oil and only 4.6% and 6.7% respectively of the corn oil fatty acids and oleic acid were recovered in the intestinal lymph(6). In rats, long chain lipids are transported from the intestine largely if not exclusively *via* lymphatic channels, since as much as 90% of the dietary lipid has been recovered in the intestinal lymph(7-9). Apparently in the dog, however, mechanisms other than the actions of pancreatic juice and bile can account for a substantial degree of fat assimilation. Investigations are planned to elucidate these mechanisms *in vivo*.

Summary. In 6 dogs deprived of both pancreatic juice and bile and fed varying amounts of olive oil or oleic acid added to a control meal, the fat assimilative deficiency was approximately 60%. It was comparable in 3 dogs with bile deficiency. In 4 dogs lacking pancreatic juice, oleic acid was absorbed as well as in normal dogs. However, the assimilative deficiency for olive oil was nearly 50%. Apparently in the dog, mechanisms other than the actions of bile and pancreatic juice can account for the assimilation of approximately 40% of the dietary fat.

The technical assistance of Robert A. Watkins is gratefully acknowledged.

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1. Annegers, J. H., *Arch. Int. Med.*, 1954, v93, 9.

2. Markowitz, J., Archibald, J., Downie, H. G., *Experimental Surgery,* Williams & Wilkins, Baltimore, 1954, 357.

3. Cohen, B. J., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 122.

4. Fowweather, F. S., Anderson, W. N., *Biochem. J.*, 1946, v40, 350.

5. Cohen, B. J., Annegers, J. H., *Gastroenterology,* 1953, v25, 67.

6. Kim, K. S., Bollman, J. L., *A. M. A. Arch., Surg.*, 1954, v69, 247.

7. Bergström, S., Blomstrand, R., Borgström, B., *Biochem. J.*, 1954, v58, 600.

8. Bloom, B., Chaikoff, I. L., Reinhardt, W., Entenman, C., Dauber, W. G., *J. Biol. Chem.*, 1950, v184, 1.

9. ———, *Am. J. Physiol.*, 1951, v166, 451.

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The Bilirubin Overload Test with Special Reference to Liver Damage Produced by India Ink.* (26527)

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Data are presented on the effects of various agents on the bilirubin overload test in the rat. This test has been used previously in the rat to study hepatic regeneration(1). It differs from the bilirubin test in man in that the amount of bilirubin administered is sufficient to overload the maximum excreting ability of the liver. Special reference will be made to liver damage due to India Ink, since the test permits ready dissociation of the action of shellac and carbon particle fractions.

Material and methods. Sprague-Dawley male rats, weighing from 120 to 400 g, were anesthetized lightly with ether. Three mg per 100 g body weight of unconjugated bilirubin (Eastman Kodak) dissolved in 0.1 M sodium carbonate were given intravenously. Serum bilirubin levels were routinely read at 3 and 30 minutes, and 1,2,4 and usually 24 hours following injection, but only the 30 minute, 1 and 2 hour points were utilized in assessing the test. Serum bilirubin was estimated by a micro-method after Malloy and Evelyn(2) utilizing 25 lambda of serum, except that the diazo reagent was modified by addition of 0.6 ml instead of 0.3 ml of sodium nitrite. Direct-reacting bilirubin was read at 1 minute. The various agents studied were administered 30 minutes prior to injection of bilirubin unless stated to the contrary (Table I). The dose of sulfobromophthalein sodium (BSP) was 3 mg per 100 g body weight and the serum levels read at 30 minutes.

In the India Ink studies, the shellac fraction was prepared by centrifugation of Higgins American India Ink at 35,000 rpm for 1 hour. Microscopy showed that it was clear of carbon particles. A carbon suspension was prepared by suspension of India Ink precipitate in a 5% gelatine normal saline solution by means of an ultrasonic generator. Since, however, complete suspension was difficult, Germantown Lamp Black was later substituted, but the results were similar. Routine paraffin and frozen sections were made of the liver and stained by standard technics. Additional animals were used to study early histological lesions.

Results. Control animals. In the normal rat, there was a rise of total serum bilirubin level to a maximum around 3 minutes after which there was a rapid fall for 2 hours, followed by a slow fall to normal at 24 hours (Fig. 1). No direct-reacting bilirubin could be detected. Repeated injections of unconjugated bilirubin at half hour intervals in 10 animals did not alter the initial rapid fall of serum bilirubin levels indicating the tremendous hepatic excretory reserve in this animal. Cannulation of the common bile duct in 10 rats showed that approximately two-thirds of the exogenous bilirubin was excreted during the first 4 hours and the remainder during the next 24 hours. Anesthesia by intraperitoneal pentobarbital did not alter the normal curve. In normal animals, no BSP serum levels were present at 30 minutes.

Experimental group. Our results are summarized in Table I. Two abnormalities were observed: 1) A delay in the fall of total bilirubin serum levels, which was significant at 30 minutes after bilirubin injection although it could be demonstrated sooner. While a single value in animals with abnormal retention might be within the upper range of values seen in control groups, differences were best appreciated by evaluation of the entire curve, since overlap at all 3 times was not seen. Analysis by the Mann-Whitney "U" test showed the differences between control and abnormal groups to be highly significant. 2) A more striking finding in animals with bilirubin retention was the invariable appearance of a direct reacting bilirubin in the serum, which reached its maximum in approximately 30 minutes and fell to normal levels at 2 to 4 hours.

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* Supported in part by grant from Nat. Cancer Inst., P. H. S.

TABLE I. Effect of Different Agents on Bilirubin Overload Test Showing Results Obtained at 30 Minutes and at 24 Hours after Administering Agent.

				30 min.				60 min.				120 min.			
	No.	Dose*	Route	Mean	Range	Significance	Direct bilirubin	Mean	Range	Significance	Direct bilirubin	Mean	Range	Significance	Direct bilirubin
Controls	20	—	—	28.2	22–33	—	0	15.5	11–19	—	0	7.0	5–10	—	0
Simultaneous BSP test	5	3.0 mg	—	26.4	21–33	N.S.	0	15.2	11–19	N.S.	0	7.0	6– 9	N.S.	0
Sodium salicylate	5	25.0 mg	I.P.	27.6	26–31	”	0	14.4	14–15	”	0	6.2	6– 7	”	0
India Ink & constituents															
India Ink	5	.2 ml	I.V.	48.2	33–60	H.S.	8.0 (5–13)	39.4	30–48	H.S.	4.2 (2–7)	29.0	25–34	H.S.	1.8 (1–3)
After 24 hr	5	*idem*		43.0	38–52	”	6.8 (6– 9)	33.2	28–41	”	2.8 (2–5)	21.6	16–29	”	0
Shellac	5	”	”	47.8	37–59	”	9.6 (5–12)	38.6	30–45	”	5.4 (2–9)	29.8	26–34	”	2.6 (1–5)
After 24 hr	5	”		40.8	34–45	”	6.4 (4– 9)	33.6	29–38	”	3.6 (1–6)	20.8	17–23	”	0
Carbon suspension	5	”	”	28.8	20–33	N.S.	0	14.6	12–17	N.S.	0	6.6	5– 9	N.S.	0
Gum acacia	5	”	I.S.	30.0	26–34	”	0	17.2	15–19	”	0	7.0	6– 9	”	0
Carbon tetrachloride	5	.06 ml	I.P.	36.0	27–41	H.S.	5.4 (4– 6)	26.2	19–30	H.S.	3.2 (2–4)	15.8	11–20	H.S.	0
After 24 hr	5	*idem*		28.0	23–32	N.S.	0	17.6	13–21	N.S.	0	8.0	7– 9	N.S.	0
Preceded by dibenzyline	5	”		35.8	33–40	H.S.	3.4 (3– 4)	22.6	18–28	H.S.	2.4 (2–3)	13.6	10–19	H.S.	0
50% ethanol	5	.6 ml	I.G.	34.0	33–37	”	4.4 (4– 5)	22.4	21–25	”	2.4 (2–3)	14.2	12–16	”	0
After 24 hr	5	*idem*		32.8	18–59	N.S.	0	15.0	8–30	N.S.	0	7.8	5–10	N.S.	0
Chlorpromazine	5	1.0 mg	I.P.	32.0	28–38	”	0	13.6	11–16	”	0	7.0	5– 9	”	0
Chloroform	5	.06 ml	”	24.2	21–28	”	0	13.8	10–19	”	0	6.4	6– 7	”	0
Choline deficient diet 21 days	5	—	—	36.2	33–39	H.S.	4.5 (4– 5)	19.0	17–21	H.S.	2.4 (2–3)	12.0	11–13	H.S.	0

* Dose/100 g body wt.

N.S. = $P > 0.10$
H.S. = $P < 0.01$

I.V. = via femoral vein
I.S. = via intrasplenic route
I.P. = via intraperitoneal route
I.G. = via intragastric route

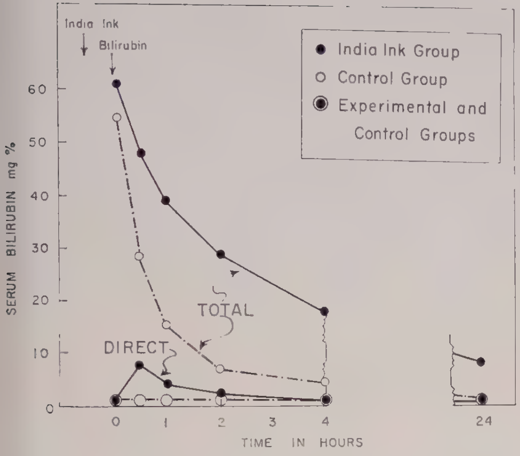
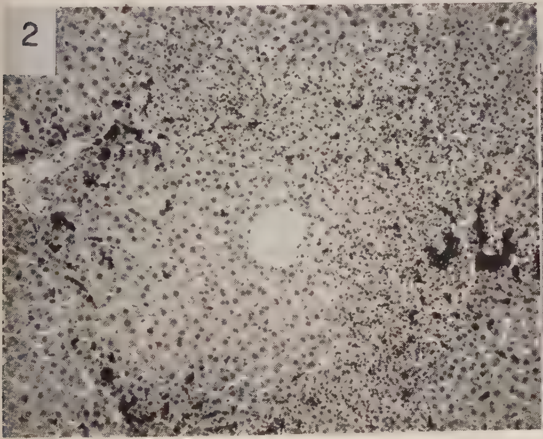


FIG. 1. Graph showing typical abnormal curve following whole India Ink administration 30 min. before inj. of bilirubin.

In animals given India Ink or shellac, total bilirubin serum levels were elevated at 24 hours and bilirubin overload indicated retention. With CCl_4 and ethanol, with one exception 24 hour serum bilirubin levels were normal and no retention or direct bilirubin could be demonstrated. Administration of India Ink, shellac, and carbon suspension to a further 22 animals by intra-splenic injection or in larger doses showed no significant differences, although histological lesions in the liver with whole India Ink tended to be more severe. Cannulation of the common bile duct in 10 rats given India Ink and 9 animals given CCl_4 showed a delay in bilirubin excretion over a 24 hour period, and a reduction in total quantity of bile excreted.

There was no correlation between degree of parenchymal damage and the abnormal bilirubin tests as indicated by studies with India Ink. Intravenous and intra-splenic injection of whole India Ink led to coagulative necrosis throughout the liver, beginning at 4 to 6 hours and reaching a maximum at 24 hours (Fig. 2). This necrosis appeared focal in the early stages, but later was predominantly periportal. It was usually associated with large intrasinusoidal masses of India Ink-laden Kupffer cells. The absence of significant necrosis in the rats given the shellac suggested that it was due to infarction following sinusoidal obstruction. Some portal edema was also present. While the shellac produced identical abnormalities in the bilirubin test, the degree of necrosis and portal edema was minimal or absent (Fig. 3). The degree of necrosis produced by the carbon suspension was much less than with whole India Ink. This was probably due to less carbon reaching the liver as much more was filtered by the lungs and spleen as compared to whole India Ink. In 2 livers after intra-splenic injection of carbon suspension, there were considerable carbon



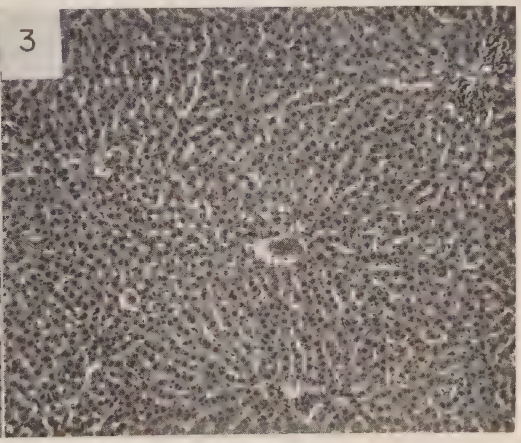


FIG. 2. Severe periportal coagulative necrosis in liver 27 hr after administration of whole India Ink. Note ink-laden phagocytes completely obstructing sinusoids at periphery of lobule. H. & E. × 120.

FIG. 3. Liver 27 hr after administration of shellac fraction of India Ink showing no significant histological abnormality. Bilirubin curve was identical to that given by whole India Ink. H. & E. × 120.

deposits in the sinusoids and Kupffer cells with some necrosis, but bilirubin tests were normal. Although typical moderate degenerative changes were present in the livers 24 hours after CCl_4, bilirubin curves were normal. Administration of dibenzyline (0.5 mg/100 g) 30 minutes prior to CCl_4 in 5 rats rad no significant effect on the test. No significant histological lesions were noted in the ethanol treated animals at 1 and 2 hours. The BSP test gave essentially similar results to the bilirubin overload test except in rats on a choline deficient diet when serum levels were normal at 30 minutes.

When BSP was administered simultaneously with bilirubin in the same syringe, no obvious bilirubin retention was observed (Table I).

Discussion. The experimental bilirubin overload test can indicate hepatic abnormalities early. It has the advantage of testing a physiological mechanism of the liver, and it may be also a more sensitive index than the BSP test as indicated by findings in choline deficient rats. It cannot be correlated with degree of liver necrosis, possibly due to the great reserve ability of the rat liver to excrete bilirubin, thus masking mild or moderate necrosis. The nature of the delayed excretion is uncertain. Due to its rapid onset and the variety of agents which produce it, it may be a non-specific vasomotor response causing anoxia as has been suggested to occur in CCl_4 poisoning. Administration of dibenzyline, however, 30 minutes prior to CCl_4 had no effect on the test. The source of the direct-reacting bilirubin is unknown. It may represent leakage into the serum from partially damaged cells, since with BSP and fluorescein there is considerable parenchymal storage prior to excretion(3). However, while there was destruction of liver cells in both India Ink and CCl_4 treated rats, direct-reacting bilirubin at 24 hours was observed only in animals treated with India Ink or shellac, and could not be correlated with histological damage. The second possibility is that it represents an extra-hepatic source of production. Such a source of conjugated bile pigment has been suggested by Hoffman *et al.*(4). However, it is difficult to appreciate why such a pigment should not appear after simple overloading. It would seem more probable to regard this pigment as hepatic in origin, but it is necessary to establish if it is a mono- or diglucuronide.

It has been shown(5,6) using the BSP test, that in the dog, the shellac fraction is responsible for the physiological activity of India Ink with the assumption that the carbon particles are harmless. It would appear, however, that the carbon particles in association with shellac may cause marked hepatic damage due to a mechanical effect. Unfortunately, we were unable to procure other fractions of the shellac from the manufacturers for testing.

Failure to demonstrate increased retention of bilirubin or BSP, when the tests were carried out simultaneously in contrast to the observations of Cantarow *et al.*(7) with prolonged infusion in dogs is probably due either to the fact that high levels of both substances were maintained only for a short period, or to the tremendous reserve of the rat liver.

Summary. The bilirubin overload test is described and the result obtained with a variety of hepatotoxic agents is presented. Hepatic injury can be demonstrated at a very early stage, but the test is of no value in indicating degree of histological liver damage. The biological effect of whole India Ink on the liver can be divided into: 1) Micro-infarction due to obstruction of the sinusoids by India Ink-laden Kupffer cells, an effect not detectable by the bilirubin overload test; and, 2) effect of the shellac which causes only minimal histological damage but which gives an abnormal bilirubin test.

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1. Weinbren, K., Billing, B. H., *Brit. J. Exp. Path.*, 1956, v37, 199.

2. Malloy, H. T., Evelyn, K. A., *J. Biol. Chem.*, 1937, v119, 481.

3. Hanzon, V., In Liver Function, Ed., Brauer, R. W., Washington, D. C., *Am. Inst. Biol. Sci.*, 1958, 281-290.

4. Hoffman, H. N., Whitcomb, F. F., Jr., Butt, H. R., Bowman, J. L., *J. Clin. Invest.*, 1960, v39, 132.

5. Zilversmit, D. B., Shore, M. L., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 442.

6. Brauer, R. W., *Gastroenterology,* 1958, v34, 1021.

7. Cantarow, A., Werts, C. W., Snope, W. J., Miller, L. L., *Amer. J. Physiol.,* 1948, v154, 211.

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Reactiveness of Fetal Pituitary to Stressful Stimuli. Does the Maternal ACTH Cross the Placenta? (26528)

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Normally the concentration of ascorbic acid in the adrenal of healthy adult animals is reduced by adrenocorticotrophin (ACTH) released from the anterior pituitary gland. The same pituitary-adrenal relationship probably exists in the fetus, since ACTH release by the fetal pituitary and responsiveness of fetal adrenals has been shown(1-4). However, the question has not been fully resolved as to whether the source of the ACTH to which the fetal adrenals respond is the fetal or the maternal hypophysis. The question of placental permeability to ACTH arises. It is generally believed that the pituitary hormones, being proteins, do not cross the placental barrier under normal conditions. Yet some authors claim that under certain experimental conditions ACTH may cross the placenta(5,6). The authors have shown that subcutaneous injection of epinephrine into embryos after the 19th day caused a significant depletion of ascorbic acid from the adrenals of the fetus(7). In this paper the effect of epinephrine injection into the fetus was determined in normal and in decapitated fetuses.

Materials and methods. All experiments were carried out on rats on the 22nd or last day of pregnancy under ether anesthesia. The sequence of operation on the embryos was from ovarian to the cervical end of one horn of the utreus, then from the ovarian to the cervical end of the other horn. First, 2 control untreated embryos were removed through a small incision in the uterine wall, blotted on filter paper and weighed on a torsion balance. After decapitation, adrenals were removed and cleaned under 10-fold magnification, pooled and weighed in a closed vessel on an automatic analytical balance. Later the ascorbic acid content of the adrenals was determined by a modification(8) of the Roe and Kuether method. The next pair of embryos was similarly treated to also serve as control. The values from these 2 pairs of embryos established the 100% value for adrenal ascorbic acid concentration for the experimental embryos of the same female rat. In all experiments 4 adrenals were pooled for ascorbic acid determination. The next 4 fetuses were decapitated as follows: A circle of stitches of a thick surgical thread was placed on the uterine wall just above the head of the fetus. An incision in the middle of this circle allowed the head to peep through. The stitches were tightened around the throat of the fetus, then the head was cut off just above the ligature. This decapitation does not disturb the normal intrauterine position of the fetus. Removal of the 4 control fetuses takes only 1 to 2 minutes. It takes about 4 minutes to decapitate the first pair of fetuses (Decapitation I) and 4 additional minutes to decapitate the second pair of fetuses (Decapitation II). When the fetuses were to be further treated, epinephrine (20 μg/fetus), or ACTH* (50 milliunits/fetus) was injected subcutaneously in aqueous solution into the fetus through the uterine wall immediately after decapitation. The remaining fetuses were left untreated to determine to what extent the adrenals were stimulated by the laparotomy of the mother as well as by the operations on and injections into the other embryos. Exactly one hour after

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* ACTH "Prolek" 1 mg contains 25 I. U.

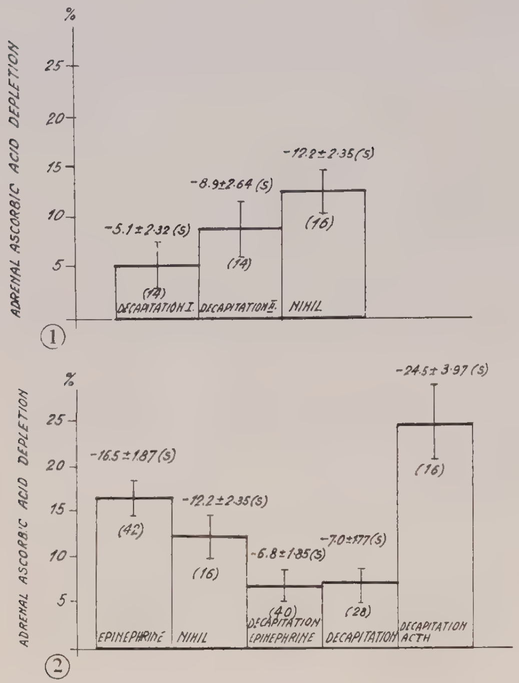

FIG. 1 and 2. Adrenal ascorbic acid depletion of 22-day-old rat fetuses. Vertical bars represent mean values, vertical lines, stand. error of means. Numbers within bars represent number of observations. S = significant depletion from control.

injection of epinephrine or ACTH under ether anesthesia, the decapitated fetuses and the remaining untreated fetuses were removed from the uterus and adrenal ascorbic acid concentration measured as above. Adrenal ascorbic acid depletions are expressed as the percentage change from ascorbic acid concentration in the adrenals of the 2 pairs of control embryos.

Results. The depletion of ascorbic acid from adrenals of the fetuses increased with time and amount of manipulation of the pregnant uterus (Fig. 1). There was only 5.1% depletion in the fetuses first decapitated (6 min), 8.9% depletion in those decapitated 4 minutes later (10 min), and 12.2% depletion one hour later in those that were untreated and killed at end of experiment (1 hour and 10 min). When 20 μg of epinephrine were injected subcutaneously into decapitated fetuses there was no further depletion of ascorbic acid (6.8% *vs.* 7.0%), (Fig. 2). When 50 mU of ACTH were injected subcutaneously into decapitated fetuses depletion was increased from 7.0% to 24.5%. In other rats, 20 μg of epinephrine were injected in fetuses without decapitation and an insignificantly greater depletion was found than in uninjected fetuses (16.5% *vs.* 12.2%).

Discussion. The failure of epinephrine, in decapitated fetuses, to cause greater depletion of ascorbic acid from the adrenals than that found in decapitated controls (6.8% *vs.* 7.0%), was expected because of the absence of the fetal pituitary. Epinephrine was injected into intact fetuses as a stressful stimulus but the data indicate that the stress of the operative conditions was sufficient to cause nearly as much depletion (16.5% *vs.* 12.2%). The much greater depletion in the intact fetuses than in decapitated fetuses (16.5% and 12.2% *vs.* 6.8% and 7.0%) proves that the fetal pituitary plays the dominant role in causing adrenal ascorbic depletion. The diminished reactiveness of adrenals of decapitated fetuses is excluded by highly significant adrenal response to exogenous ACTH (24.5%). The small, but significant depletion of adrenal ascorbic acid in decapitated uninjected fetuses (7.0%) may be caused either by maternal ACTH which crossed placenta or by fetal ACTH discharged from the fetal pituitary before its removal. The latter possibility is somewhat supported by the evident tendency of greater depletion of adrenal ascorbic acid by the pair of fetuses decapitated later in relation to those operated first (8.9% *vs.* 5.1%), and especially by the greater depletion obtained in intact fetuses (12.2%). If maternal ACTH were the cause of the depletion, then the same depletion should be found in all 3 groups. It may be concluded that the ascorbic acid depletion from the adrenals of the fetuses is due to fetal ACTH and not to ACTH from blood of the mother.

Summary. Stressful stimulus in intact fetuses, but not in decapitated fetuses, caused adrenal ascorbic acid depletion. ACTH injection into decapitated fetuses was followed by considerable adrenal ascorbic acid depletion. These data support the concept that the placenta is not permeable to ACTH.

1. Jost, A., *Recent Progress in Hormone Research,* 1953, v8, 379.

2. ———, *Cold Spring Harbor Symposia on Quantitative Biology,* 1954, v19, 167.

3. Moog, F., *Comparative Endocrinology,* John Wiley & Sons, Inc., N. Y., Ed. A. Gorbman, 1959, p. 624.

4. Wells, L. J., *ibid.,* 444.

5. Jones, J. M., Lloyd, C. W., Wyatt, T. C., *Endocrinol.,* 1953, v53, 182.

6. Knobil, E., Briggs, F. N., *ibid.,* 1955, v57, 147.

7. Milkovic, K., Milkovic, S., *Arch. internat. Physiol. Bioch.,* 1958, v66, 534.

8. Abelson, D., Baron, D. N., *Lancet,* 1952, vII, 663.

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Effects of Conjugated Bile Acids on *in vivo* Cholesterol Metabolism in the Mouse.* (26529)

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Considerable data have accumulated on the effects of various bile acids on rates of cholesterol synthesis in different tissues(1,2). If one couples these data with the finding that certain bile acids are principal end products of cholesterol metabolism(3,4), it appears most probable that these substances are active in the control of certain phases of cholesterol metabolism. However, free bile acids—not the glycine and taurine conjugates—were employed in this work. It is, therefore, possible that some of the observed effects were due to exhaustion of the supply of amino acids used in conjugate formation; *viz.,* glycine, serine, and cysteine. A deficiency of any of these could have a serious effect on cholesterol metabolism and on metabolism in general. To check this possibility, the glycine and taurine conjugates of a number of bile acids were synthesized and their effects on cholesterol metabolism compared with those of the free bile acids.

Methods. The conjugates of cholic, deoxycholic, and hyodeoxycholic acid were synthesized according to the method of Norman(5), with some modification. The purity of the conjugates was checked by paper chromatography(6) and elementary analysis.

Elementary Analysis of Bile Acid Conjugates

		Calc.	Found
Glycocholic acid	N	3.01	3.25
Sodium Taurocholate	S	5.96	5.70
Glycodeoxycholic acid	N	3.12	3.32
Taurodeoxycholic "	S	6.15	6.04
Glycohyodeoxycholic acid	C	69.45	70.0
	H	9.64	10.37
	N	3.12	3.07

One hundred female albino mice of the Webster strain, weighing 21-26 g, were placed on a cholesterol free diet(7) for a 2-week period. They were then divided into 10 equal groups and diets supplemented as shown in Table I. All diets were fed *ad lib.*

After 3 weeks each animal received an intraperitoneal injection of 5 μc sodium acetate-1-C^{14}. One hour later the animals were sacrificed and livers removed for analysis of cholesterol and cholesterol-x-C^{14}(4). Cholesterol isolated from tissues was purified through the dibromide(8).

Results and discussion. Effects of free bile acids and of bile acid conjugates, are, in most cases, qualitatively similar (Table I). The conjugates of cholic and deoxycholic acids raise liver cholesterol levels to a greater extent than the free bile acids, but they do not have this increased effect on acetate-1-C^{14} incorporation rates. However, these rates were lowered one-sixth to one-tenth by free cholic and deoxycholic acid, so that any further lowering would be difficult to detect with any certainty because of high deviations.

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* This work was supported in part by Research Grant from Nat. Inst. Health.

Extract of hog bile was supplied by Wilson Laboratories, Chicago, Ill.

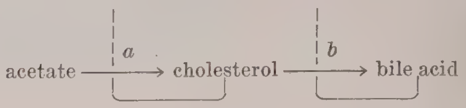
TABLE I. Effect of Bile Acids and Bile Acid Conjugates on Mouse Liver Cholesterol Levels and Synthesis Rates.

Dietary groups*		Total liver cholesterol, mg/g†	Cholesterol-x-C^{14} activity, counts/min./g tissue
I	Controls	5.32 ± 1.24	1176 ± 312
II	Cholic acid	6.73 ± 1.47	145 ± 64
III	Glycocholic acid	9.33 ± 1.61	241 ± 91
IV	Sodium taurocholate	8.52 ± 1.54	386 ± 205
V	Deoxycholic acid	4.52 ± 1.35	201 ± 80
VI	Glycodeoxycholic acid	8.41 ± 2.23	201 ± 109
VII	Sodium Taurodeoxycholate	7.86 ± 1.64	265 ± 142
VIII	Hyodeoxycholic acid	2.97 ± .39	3374 ± 909
IX	Glycohyodeoxycholic acid	3.28 ± .81	6827 ±1890
X	Hog bile extract (45% glycohyodeoxycholic acid)	3.41 ± .53	3286 ±1130

* Supplements were fed at level of 0.5% of diet.
† Wet wt.

The results for sodium taurocholate (Group IV) are not in accord with those reported by Siperstein(9), but his experimental conditions were different since he employed rats rather than mice. As previously pointed out(10), the effects of various bile acids exhibit a high degree of species specificity. Moreover, the effect of sodium taurocholate on lowering the rate of acetate-1-C^{14} incorporation, atlhough pronounced, was significantly less than that of free cholic acid or its glycine conjugate.

Although cholesterol levels and synthesis rates are inversely related, this relationship is not quantitatively significant. For example, liver cholesterol levels in mice treated with hyodeoxycholic acid (Group VIII) and glycohyodeoxycholic acid (Group IX) are not significantly different; however, cholesterol synthesis rate for Group IX is double that for Group VIII. In another experiment previously unreported, liver cholesterol level of control mice was 7.25 mg/g while that of mice on a diet supplemented with 3% corn oil was 3.93 mg/g. This represents a 45% decrease in cholesterol level, but the cholesterol synthesis rate was the same in both groups. These facts are somewhat surprising if the double feedback mechanism of action of the bile acids is valid. An increase in bile acid concentration blocks reaction sequence *b* causing cholesterol to accumulate. This blocks sequence *a*, resulting in diminished incorporation of acetate into cholesterol.



Siperstein(9) has recently found evidence that it is not cholesterol *per se* which inhibits its own synthesis, and this may explain the lack of a quantitative relationship. Furthermore, we may be approaching limiting conditions in these experiments.

Although the results show that the effects of bile acids are not mediated through exhaustion of the supply of certain amino acids, they do not show whether conjugated bile acids or free acids are responsible inasmuch as some hydrolysis of the conjugated acid by intestinal flora would be expected during hepato-intestinal recirculation.

Cholesterol-x-C^{14} isolated from various groups was converted to the dibromide, regenerated and recrystallized. There was more or less loss of activity (Table II) in Groups I through VII. The activity of controls and of Groups VIII and IX changed only slightly. Thus the overall results are somewhat enhanced and the conclusions remain valid.

Summary. Free and conjugated bile acids had qualitatively similar effects on mouse liver cholesterol levels and *in vivo* cholesterol-x-C^{14} synthesis rates. The effects of free bile

TABLE II. Specific Activity of Cholesterol before and after Dibromide Purification, Counts/Min./mg.

Dietary groups*		Before	After
I	Controls	123	112
II	Cholic acid	23	17
III	Glycocholic acid	24	13
IV	Sodium taurocholate	51	43
V	Deoxycholic acid	78	73
VI	Glycodeoxycholic acid	30	19
VII	Sodium taurodeoxycholate	54	38
VIII	Hyodeoxycholic acid	245	240
IX	Glycohyodeoxycholic acid	89	88

* Supplements were fed at level of 0.5% of diet.

acids on cholesterol metabolism are not due to the exhaustion of amino acids used in formation of glycine and taurine conjugates.

1. Beher, W. T., Baker, G. D., *Am. J. Physiol.*, 1959, v197, 1339.
2. Beher, W. T., Anthony, W. L., Baker, G. D., PROC. SOC. EXP. BIOL. AND MED., 1959, v102, 317.
3. Siperstein, M. D., Jayko, M. E., Chaikoff, I. L., Dauben, W. G., *ibid.*, 1952, v81, 720.
4. Beher, W. T., Baker, G. D., Anthony, W. L., *Am. J. Physiol.*, 1960, v199, 736.
5. Norman, A., *Arkiv. f Kemi*, 1955, v8, 331.
6. Sjövall, J., *ibid.*, 1955, v8, 317.
7. Beher, W. T., Baker, G. D., Anthony, W. L., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 385.
8. Frantz, I. D., Davidson, A. G., Dulit, E., Mobberley, M. L., *J. Biol. Chem.*, 1959, v234, 2290.
9. Siperstein, M. D., *Am. J. Clin. Nutrition*, 1960, v8, 645.
10. Beher, W. T., Anthony, W. L., Baker, G. D., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 531.

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A Method for Transplantation of the Rabbit Kidney.* (26530)

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The study of kidneys transferred from one dog to another has provided important information on the transplantation reaction. Hume(1) has recently reviewed the work of Carrel, Williamson, Dempster, Simonson and others in this field. However, extensive employment of the kidney in transplantation research has been limited because currently available surgical technics necessitate the use of large animals such as dogs. The expense of preparation, maintenance and sterile surgical procedures required in dogs has discouraged their widespread use in such studies. Some of these difficulties can be eliminated by transplanting organs in the rabbit. This report describes a method which for the first time makes possible chronic homologous transplantation of the rabbit kidney by vascular anastomosis. This method has been employed to show the histological changes in the rabbit kidney during the rejection process.

Method. Selection and preparation of host. The recipients of kidney transplants were randomly bred albino rabbits, weighing about 4.0 kg. They were anesthetized by intravenously administered Nembutal (25 mg/kg) and placed on their backs with legs securely tied to the operating table. In this position, the retraction of the rabbit's neck often produces difficulties with respiration. To overcome this problem, 100% oxygen was administered under the surgical drape which covers the animal's head. The drape acted as a tent which partially contained the gas.

An intravenous drip containing 30 mg Nembutal and 2.5 mg heparin per 100 cc saline was connected to a peripheral ear vein. The rabbit received about 75 cc of this solution during a 2½ hour period. The heparin was given during surgery because it appeared to help prevent clotting at sites of the anastomoses. No additional anticoagulant was given to the host after operation.

A clean surgical procedure was used, but sterile precautions were not observed. The carotid artery and jugular vein on one side of the recipient rabbit's neck were isolated, clamped and divided, then their distal ends were tied with silk ligatures. Their proximal stumps were later anastomosed to the donor vessels.

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* This work was supported in part by grants from USPHS and the Josiah Macy, Jr. Foundation. The authors thank Dr. Bernard C. Wexler for help in preparation and evaluation of histologic material, and Mrs. Barbara Bouldin and Miss Kathleen Robb for excellent technical assistance.

† This work was carried out during the tenure of an Advanced Research Fellowship of American Heart Assn. to Dr. Nathan.

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Selection and preparation of donor. Kidney donors were young, outbred New Zealand or Dutch rabbits, weighing one-half to one kilogram. Size of the donor and recipient was selected so that the vessels to be connected would be approximately of the same diameter. The left kidney, renal vessels, and ureter were isolated through an abdominal incision. Because of the lower position of the left kidney and the length of its renal vein, it is more easily transplanted than the contralateral organ. A few centimeters of abdominal aorta and vena cava *above and below* the origin of the renal vessels were dissected free. All of the branches, except the renal vessels, were tied and divided. The donor was then given an intravenous injection of one mg of heparin.

Preparation of anastomoses. The isolated aorta and vena cava were next tied off about 2 cm above and below the origin of the renal vessels to obtain adequate T-sections. A small incision just above the distal ligatures on the major abdominal vessels permitted insertion of short lengths of plastic tubing about one-half the diameter and 5 cm longer than the isolated abdominal vessels. The plastic tubes were pushed forward until they reached the anterior ligature. Proximal ends of aorta and vena cava were then divided just below their anterior ties. The elasticity of the vessels caused them to shorten so that the plastic tubes protruded beyond the cut edges. Ligatures were then tied around aorta and vena cava below the origin of the renal vessels to prevent the plastic tubes from slipping out. The aorta and vena cava were severed between the 2 distal ties. When the vessels were thus prepared and the ureter freed and cut at the bladder~~, the~~ kidney with all its attached structures was removed from the donor.

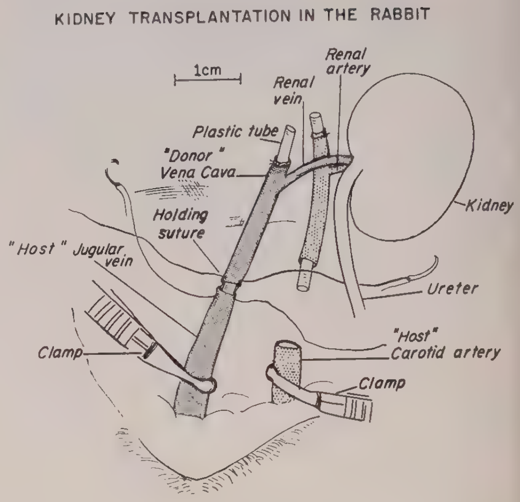

FIG. 1. Schematic drawing of part of procedure for transplantation of rabbit kidney demonstrates the homotransplant placed in neck of recipient just prior to suturing the vessels. Later, the ureter will be exteriorized through a stab wound in the skin near top of kidney.

The donor kidney placed in the neck of the host is shown in Fig. 1. In the schematic drawing, the distal ties (not labelled) which hold the plastic tubing are located on the abdominal vessels above the renal artery and vein. Two holding sutures using 6-0 silk were placed so that the cut ends of the vena cava and jugular vein could be approximated. Next, the protruding plastic tube in the vena cava was inserted into the cut end of the host's jugular vein. Note that the tubing prevents the collapse of both donor and recipient vessels. The veins were then connected by a continuous silk suture and arteries were similarly intubated and anastomosed.

When the anastomosis had been completed, the distal ties holding the plastic tubes were cut and the tubes removed through the open ends of the vessels. The aorta and vena cava were next tied just above the renal vessels (Fig. 1). When the vascular clamps were removed, the blood of the recipient flowed from its carotid artery into the aorta and renal artery of the donor. The return flow is through the donor's renal vein and abdominal vena cava to the jugular vein of the host.

The recipient's skin was closed over the kidney, and the ureter of the transplant was brought out through a separate stab wound. A small plastic tube was inserted into the ureter and tied, and thus served as a catheter. An antibiotic salve was applied to keep the surface of the exposed ureter in good condition.

Preparation of autotransplants. As a con-

TABLE I. Homologous Rabbit Kidney Transplants.

Exp. No.	Biopsy on POD	Urine flow observed on POD	Transplant removed POD	Ischemia (min.)
1	10, 14, 17	16	17	99
2	7, 14, 21, 28	19	28	54
3	4, 15, 22	19	22	60
4	5, 7	5	7	32
5	9	—	9	39
6	2	1	2	175
7	4	3	4 (host died)	50
8	—	3	4 (" ")	110
9	—	3	7	153
10	—	3	4 (" ")	64
11	5	3	5	60
12	7, 13	3	13	55

trol for the homotransplants, 2 autotransplants were prepared in which the renal artery and vein of the host's kidney were connected to vessels in his neck. For this procedure it is not possible to use the method described above. However, plastic tubes were employed during part of the operation and were removed through the anastomotic site after the major portion of the connecting suture line was completed(2). The small size of the renal artery and vein make this suturing difficult.

To obtain biopsies, several days or weeks after surgery, a small incision was made in the skin over the kidney to expose the transplant. Specimens of the tissue were removed and prepared for histologic study. Tissue sections were all stained with hematoxylin and eosin.

Results and observations. Table I summarizes results of 12 consecutive kidney transplantations in rabbits. Most of the transplants were removed 2 to 9 days after operation in order to compare the time-course of rejection with well documented observations on dogs(1) in which the major histologic features of kidney rejection occur within a week. Three of the transferred kidneys were allowed to remain in place for 17 to 28 days. Urine flow was observed from these organs for more than 16 days after surgical procedures.

Table I shows that the period of renal ischemia required for operation is about one hour. In some instances fewer than 40 minutes was sufficient to complete the operation. Three of the 12 animals referred to in Table I died for unknown reasons 4 days after the transplantation.

The structural changes observed in the biopsy material can be summarized as follows: Within 4 days after grafting there is a definite perivascular accumulation of white cells (Fig. 2). These continue to infiltrate the tissue until the major portion of the parenchyma is no longer visible. By 7 days after transplantation, the outer layers of the cortex contain

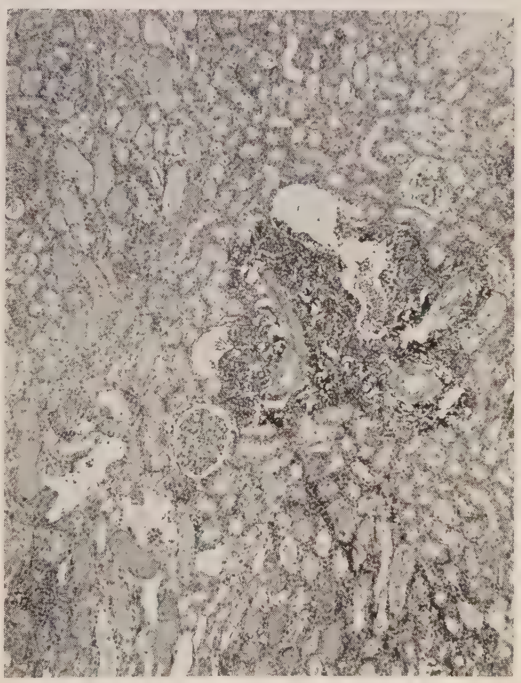

FIG. 2. Perivascular infiltrate in rabbit kidney can be observed 4 days after transplantation. Casts are present in many of the tubules. (75×)

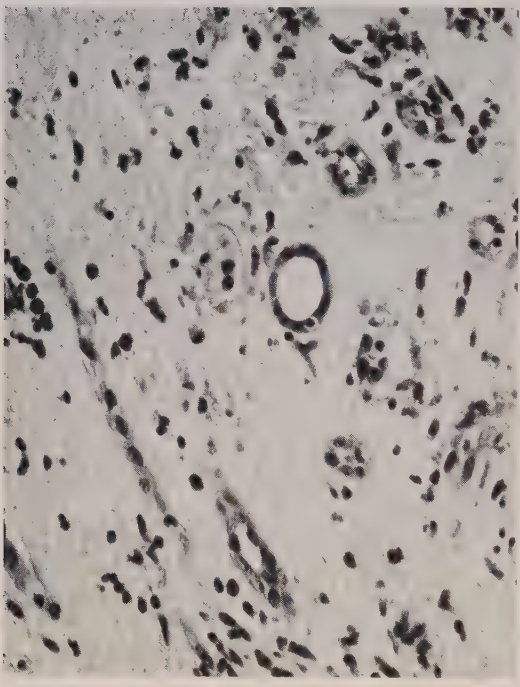

FIG. 3. Section from cortex of a kidney 28 days after homologous transplantation shows surviving tubules. (250×)

only a few viable cells.

Part of the blood supply of the transplanted kidney remains intact as long as 3 weeks after operation. In one animal the graft still bled when cut to obtain biopsy material 28 days after transfer of the kidney. Fig. 3 shows some surviving tubules in the cortex of this transplant on the 28th postoperative day. In some unpublished experiments we have shown that the total function of these relatively long-term transplants is not sufficient to keep the host alive when its own kidneys are removed.

In contrast, the autotransplanted kidneys showed a normal tissue pattern. Twenty days after the operation, the second kidney was removed from one of the animals. The host survived in good condition until 10 days later, when the autotransplant was recovered for histologic examination. Totally nephrectomized rabbits die of uremia within 3 days. Also, in the second autotransplant, this single kidney alone was sufficient to maintain the host for more than 2 weeks.

In addition to the 2 autotransplants mentioned above, the kidneys in 2 other rabbits were subjected to one hour periods of ischemia during which the renal pedicle was clamped. Upon removal of the clamps, the kidneys were allowed to remain *in situ*. Four days later tissue from these organs was examined for damage due to ischemia. Some dilated tubules and casts were seen in these sections; however, no cellular infiltrate was evident. The histological appearance in the homologous transplants described above appears to be due to the specific rejection process.

Discussion. The biopsy sections show that rejection in the rabbit is similar to that in the dog and suggests that the rabbit can be substituted for the dog in many transplant studies. We observed the following differences between kidney transplants in the dog and rabbit: Urine secretion may continue in the rabbit for almost 3 weeks after transplantation; the dog kidney almost invariably stops in less than 10 days. Some blood may flow through the rabbit kidney as long as 4 weeks after operation; in the dog, the major blood vessels tend to thrombose in less than 10 days. It is conceivable that the special renal vasculature in the rabbit may permit blood to flow after the major portion of these vessels has been destroyed. This suggestion is supported by the observation that homologous skin grafts in our rabbits are uniformly rejected in less than 12 days.

Although the cellular mechanism of rejection in most species appears to be similar, important differences in *rate* of kidney transplant rejection have become apparent from comparative studies. In contrast to the sudden and complete destruction of renal homotransplants in the dog, the present work with the rabbit demonstrates that some nucleated cells can survive in an immunized animal for more than 3 weeks, even within an organ which has been largely rejected. Homotransplants of human kidneys also have been known to survive for several months(3,4). Preliminary studies in our laboratory of renal transplants between outbred rats of the Sprague-Dawley strain show some viable cells more than 50 days after operation. The abrupt destruction of the dog kidney transplants is probably caused by the loss of their main channels of renal blood supply. On the other hand, in the rabbit, rat and man, certain parenchymal cells which have somehow retained their vascularity, may be rejected very slowly. The importance of the blood supply for rejection of skin grafts has been stressed by Conway and Stark(5) and Rolle, Taylor and Charipper(6).

The surgical procedures developed for transplantation of the rabbit kidney can be adapted to transfer other organs such as segments of intestine or adrenal glands. Wherever a T-section of vessels is available, a plastic tube can be used in the operation to maintain their patency. This simplifies suturing and also insures against the possibility of accidental closing of the lumen.

Summary. Homologous transplants of the rabbit kidney have been carried out by a procedure in which vascular anastomoses of the donor aorta and vena cava were made to the carotid artery and jugular vein of the host. During the operation, plastic tubes were employed to maintain the patency of the vessels. These tubes were removed after completion of the anastomoses. Some nucleated cells can survive 4 weeks in homologous kidney transplants. The destructive process in the rabbit kidney is considerably slower than in the dog. It is suggested that the breakdown in the vascular supply to the transplant may be important in the rejection process.

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1. Hume, D., in: *Transplantation of Tissue,* 1959, Volume II, ed. by Lyndon A. Peer. The Williams and Wilkins Co., Baltimore, pp. 484-536.

2. Snyder, C. C., Knowles, R., Mayer, P. W., Hobbs, J. C., *J. Plastic & Reconstruct. Surg.,* 1960, v26, 251.

3. Miller, B. F., *Fifth Macy Conference on Renal Function,* 1953, ed. by Stanley E. Bradley, Madison Printing Co., N. J., pp. 70-113.

4. Hume, D. M., Merrill, J. P., Miller, B. F., Thorn, G. W., *J. Clin. Invest.,* 1955, v34, 327.

5. Conway, H., Joslin, D., Rees, T. D., Stark, R. B., *J. Plastic & Reconstruct. Surg.,* 1953, v12, 74.

6. Rolle, G. K., Taylor, C., Charipper, H. A., *J. Cell. & Comp. Physiol.,* 1959, v53, 215.

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Inhibition of Cytopathic Action of Salisbury Virus by Antiviral Agent 1758.*

(26531)

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In recent tissue culture studies in work related to the common cold, we have tested the activity of an antiviral agent against the cytopathic action of a Salisbury virus derived from a human cold. The reports of the work of Dr. Andrewes' group in Mill Hill and Salisbury, England(1,2,3), have renewed our interest in cold viruses. Coincidentally, we have experimented with antiviral substances from penicillium molds which demonstrated a considerable spectrum of activity in mice when virus and antiviral agent were administered by separate routes of injection(4,5,6). We are reporting here the results of tests of successful inhibition of the cytopathic action of a Salisbury virus by a penicillium antiviral agent called "1758".

Materials. Salisbury virus H.G.P. was supplied to us as an ampoule of dried material by Dr. C. H. Andrewes. Properties of this virus have been described(1,2,3). H.G.P. virus was typed during this work with rabbit antiserum of a titer of 1-16 made with an early pool of virus. Antiviral agent 1758 was supplied to us as partially concentrated broth by Lilly Research Laboratories. This agent was derived from tank cultures of Penicillium stoloniferum (group Brevi-Compactum), and was first detected in the Lilly Labs (Powell, Culbertson, Hoehn, and McGuire, unpublished) using the technic found useful in detecting antiviral agent 8450(4). Antiviral

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agent 1758 may be of more interest than 8450, since it can be prepared from tank growth, while 8450 must be prepared from shake flask growth by a tedious procedure. Tissue culture for growing virus H.G.P. was second generation or secondary monkey kidney cell cultures as the Andrewes group have described. The virus phase has utilized the low bicarbonate content replacement medium in tubes in roller drums placed at 33°C, both of these conditions comprising the standard Andrewes technic.

Procedure. When reasonably confluent monolayers of these second generation monkey kidney cells were ready, antiviral agent 1758 was either (a) put directly on the cells and the tubes rotated 10 minutes, then replacement medium added and tubes rotated again, or (b) put into the replacement medium as added to the tubes and these rotated. Twenty-four hours after addition of 1758, H.G.P. virus was seeded into all tubes following another replacement of medium, and tubes were rotated again at 33°C. Titers of H.G.P. virus attained in tubes without 1758 were generally over 10^{-4} but under 10^{-5}. Activity of 1758 as judged by the technic used in previous mouse tests(4) was as follows: Of 10 mice injected intraperitoneally with consecutive 0.5 ml doses of 1758 diluted 1-5 at 8 A.M., noon, and 4 P.M. on day 1 and 8 A.M. of day 2 (4 doses in all), and injected subcutaneously at noon of day 2 with 100 LD_{50} of Semliki Forest virus, 4 mice died on days 5, 6, 6, and 8 respectively, and 6 mice survived and were normal on day 14.

In addition to tissue culture tubes treated with 1758 and subsequently seeded with H.G.P. virus, control tubes with tissue alone, tissue and 1758, and tissue and virus but without 1758 were included. Daily microscopic observation of all tubes was made to detect cytopathic effects of the virus. In general there was little difference between the (a) and (b) procedures above, so the (b) procedure was favored, *i.e.*, putting 1758 in the replacement medium with another replacement of medium and addition of virus 24 hours later.

Results. Application of antiviral agent 1758 to otherwise susceptible tissue culture prevented the appearance of cytopathic effects of H.G.P. virus seeded into the cultures 24 hours later. Early tests utilized 0.1 ml of 1758 diluted 1-10 in replacement medium and 0.1 ml of a 10^{-3} dilution of H.G.P. virus into 4 roller tubes, and these tubes showed no cytopathic effects. This inoculum of virus alone in 4 tubes produced complete damage in 5 days, while the 1758 alone in 4 tubes appeared to be without toxicity. Subcultures of the tubes in which virus damage to cells was prevented by 1758 into new tissue culture tubes without 1758 showed reappearance of virus and damage to the cells on days 5 and 6. The 1758 appears not to destroy or neutralize virus, but rather to act on the tissue culture cells in such a way that virus does not damage them. The antiviral agent after being boiled 5 minutes did not do this. These effects are similar to results of Hull and Lavelle for antiviral agent 8450 and the 3 types of poliovirus(7). Table I shows the results of a somewhat enlarged test including several dilutions of 1758 and different dilutions of H.G.P. virus, all dilutions being made in replacement medium. Cytopathic action of a 10^{-3} dilution of H.G.P. virus was inhibited by increasing dilutions of 1758 up to 1-10. A dilution of 1-16, however, was ineffective. An intermediate dilution of 1758 of 1-8 also inhibited the cytopathic action of virus diluted 10^{-2} and 10^{-4}. 1758 was not toxic to the cells. Boiled 1758 did not inhibit cytopathic action of the virus. Also, when cytopathic action of H.G.P. virus was inhibited by 1758, subcultures of these tubes showed reappearance of the virus and cytopathic effects. The antiviral agent thus appears again to render the cells insusceptible to virus action for a time, without neutralization of the virus. In an additional test parallel with that shown in the upper 8 lines of Table I the (a) variation in technic referred to above, *i.e.*, putting the 1758 on the cells directly and rotating the tubes 10 minutes, then adding the new replacement medium and rotating again, gave the same end point of action as that shown in the Table. Both technics showed 1758 diluted 1-10 was effective, while 1-16 was not effective. In all these tests duplicate tubes of tissue culture were used.

TABLE I. Effect of 1758 on Cytopathic Action of H.G.P. Virus.

Dilution of 1758	Dilution of H.G.P.	Cytopathic readings	Cytopathic reading: Subcultures
Undiluted	10^{-3}	— —	0
1 - 2	10^{-3}	— —	0
1 - 5	10^{-3}	— —	0
1 - 8	10^{-2}	— —	+ +
1 - 8	10^{-3}	— —	+ +
1 - 8	10^{-4}	— —	+ +
1 - 10	10^{-3}	— —	0
1 - 16	10^{-3}	+ +	0
None	10^{-2}	+ +	0
”	10^{-3}	+ +	0
”	10^{-4}	+ +	0
Undiluted	None	— —	0
1 - 2	”	— —	0
Undil. (boiled 5 min.)	10^{-2}	+ +	0
Idem	10^{-3}	+ +	0

Legend: + indicates complete damage to cell layer usually starting on day 5 or 6 and rapidly progressing. — indicates no cytopathic effect. 0 indicates not done. 1758 and H.G.P. were added in volumes of 0.1 ml.

In additional tests using a 10^{-2} dilution of H.G.P. virus throughout, the titer of 1758 was 1-5, instead of 1-10 as shown in Table I. A dilution of 1-8 almost completely, and a dilution of 1-10 partially, inhibited cytopathic action.

Our detection of active virus (lines 4, 5, and 6, Table I) after 10 to 14 days at 33°C in tissue culture protected by 1758 is compatible with some earlier unreported tests we have done on the kinetics of thermoinactivation of H.G.P. virus.

Discussion. The results reported here indicate that an antiviral agent provisionally called 1758, first detected in mouse screening tests and having chemoprophylactic action in mice similar to antiviral agent 8450, inhibits the cytopathic action of Salisbury virus H.G.P. As far as we have noted, the virus itself is not affected directly, but the tissue culture cells are made refractory for an undetermined period, at least 14 days, to the cytopathic action of the virus. In this type of antiviral action as well as in the results of mouse tests, antiviral agents 1758 and 8450 are somewhat similar. Dr. R. N. Hull has recently told us that 1758 like 8450 inhibits cytopathic action of poliovirus. Both these agents are products of penicillium molds. Since 8450 has appeared similar in spectrum to Helenin(6), also a penicillium product, it follows that 1758 is likely similar in this respect to Helenin. It is possible that, due to tank growth of 1758, more ample supplies may expedite further work.

Summary. It has been found that an antiviral agent called 1758, and somewhat similar to agent 8450 in antiviral action in mice, exhibits demonstrable inhibition of the cytopathic action of Salisbury virus H.G.P. This antiviral agent is of penicillium origin, and virus H.G.P. is one of several strains reported of common cold origin.

Addendum: See report of 1758 as Statolon in Probst, G. W. and Kleinschmidt, W. J., *Federation Proceedings*, 1961, v20, 441.

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1. Tyrell, D. A. J., Hitchcock, G., Bynoe, M. L., Periera, H. G., Andrewes, C. H., *Lancet*, 1960, v1, 235.
2. Hitchcock, G., Tyrell, D. A. J., *ibid.*, 1960, v1, 237.
3. Tyrell, D. A. J., Parsons, R. *ibid.*, 1960, v1, 239.
4. Powell, H. M., Culbertson, C. G., McGuire, J. M., Hoehn, M. M., Baker, L. A., *Antibiotics and Chemotherapy*, 1952, v2, 432.
5. Powell, H. M., Culbertson, C. G., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 161.
6. Powell, H. M., Culbertson, C. G., *Antibiot. Ann.*, 1953-54, 147.
7. Hull, R. N., Lavelle, J. M., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 787.

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Effect of Intraluminal Pressure on Enterochromaffin Cells in the Rat Duodenum.* (26532)

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It is generally agreed that, in mammals, the mucosa of the gastrointestinal tract is a major site of 5-hydroxytryptamine (5-HT) production(1,2,3). Furthermore, there is considerable experimental evidence supporting the hypothesis that the enterochromaffin cells (E.C. cells) within the mucosa are the principal, if not the sole source, of this amine (4,5,6). The pharmacological activity of 5HT has been well described in a wide variety of organs and tissues, in both man and animals, but as yet its physiological role has not been elucidated.

Bülbring and Crema(7) have shown that 5HT may be important in the peristaltic activity of the intestinal tract. Their studies in guinea pigs indicate that 5HT is released from the intestinal mucosa in response to increased intraluminal pressures. They suggest that the enterochromaffin cells might be neurosecretory cells in which "deformation leads to the release of 5HT."

The present study was undertaken to determine if histochemical alterations occur in the enterochromaffin cells of the duodenal mucosa of rats in response to increased intraluminal pressures.

Methods. White Carworth rats (200-400 g) were anesthetized with intraperitoneal 2% sodium pentobarbital (0.2 cc/100 g). The abdominal cavity was opened and a segment of duodenum, 3-4 cm in length was isolated by transection. The mesentery of the isolated segment remained intact. The proximal and distal ends of the segment were carefully cannulated with small glass cannulae. The distal cannula was connected to a water manometer to record the intraluminal pressure in the isolated bowel. The proximal cannula was connected to a sphygmomanometer bulb to permit introduction of air into the bowel and to maintain the desired luminal pressure. The isolated duodenal segment was then subjected to a predetermined pressure (20-80 mm H_2O) by insufflation of air and the pressure maintained for varying intervals of time (10-36 minutes). Portions of the experimental segment of duodenum were then quickly excised and placed immediately in 10% formalin. Control tissue was obtained from duodenum adjacent to the isolated segment. Proximal and distal sections of the duodenum were used alternately as experimental and control tissues, to compensate for possible differences in the enterochromaffin cell population at different levels of the duodenum. A total of 10 rats was used.

Following fixation, the experimental and control tissues were dehydrated with an ascending series of alcohols, cleared in benzene, infiltered and embedded in paraffin. Sections of experimental and control tissues were cut at 4 μ and mounted on the same slide. These sections were deparaffinized with xylene, and hydrated with a descending series of alcohols to water. The Gomori-Burtner methenamine silver method(8) was used for identification of the argentaffin cells.

Five millimeter lengths of the stained tissue were marked off with India ink. The enterochromaffin cells within the 5 mm lengths of tissue were then counted by independent observers and recorded. All slides were coded until the E. C. counting was completed.

Results. The results shown in Table I indicate a significant decrease in number of recognizable enterochromaffin cells in the mucosa subjected to increased intraluminal pressure as compared to controls ($P<0.0001$). When pressures of 80 mm of H_2O were maintained for longer than 20 minutes the resultant edema and ecchymosis rendered the tissue unsuitable for study. Tissue sections analyzed in this report demonstrated satisfactory preservation of histological detail.

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TABLE I. Enterochromaffin Cell Counts in 5 mm Duodenal Mucosa at Varying Pressures and Times.

No. of exp.	Pressure, mm of H_2O	Time, min.	Enterochromaffin cell counts/5 mm mucosa		
6 Control	—	20	13	11	12
Exp.	20	20	7	6	5
12 Control	—	20	13	9	10
Exp.	20	20	7	4	5
9 Control	—	25	10	11	10
Exp.	20	25	5	5	4
13 Control	—	25	9	6	7
Exp.	20	25	9	6	6
4 Control	—	30	11	9	10
Exp.	40	30	6	5	5
5 Control	—	30	11	9	10
Exp.	40	30	7	5	6
10 Control	—	30	11	11	13
Exp.	40	30	5	5	5
11 Control	—	30	15	11	11
Exp.	40	30	7	5	6
7 Control	—	10	11	9	8
Exp.	80	10	9	6	5
8 Control	—	20	7	5	4
Exp.	80	20	4	4	3

There was no apparent correlation between amount of pressure and/or length of time the pressure was maintained and extent to which the E.C. cell counts were reduced.

Discussion. It is concluded from these observations that there is a diminution in number of recognizable E.C. cells in the duodenal mucosa of rats following increased intraluminal pressure. These findings provide histochemical support for the hypothesis that pressure is one mechanism for mediating the release of 5HT from intestinal mucosa.

A reduction in actual number of E.C. cells per unit tissue is not inferred from these findings. They simply indicate that pressure upon the mucosa has resulted in a release of the argentaffin positive material (presumably 5HT) from E.C. cells, making recognition impossible by the histochemical technic employed.

It may be significant that within the range of pressures used, the mucosa was never found completely devoid of E.C. cells, suggesting that after an initial period of accelerated release, rate of formation of 5HT and its release were in equilibrium. It is noteworthy, that in guinea pigs, intraluminal pressures of 20-40 mm of H_2O maintained for 3 hours, did not deplete 5HT content of the mucosa(9).

Additional histological evidence supporting the concept that pressure and peristalsis may be related to release of 5HT by the E.C. cells may be found in Pearse's(10) observations in intestinal tuberculosis and Crohns disease. In cases where the bowel had become rigid he observed an increase in number of argentaffin cells with an increase in size and number of granules they contained. In our study, for lack of suitable methods of quantitation no attempt was made to document the degrees of granulation in the various cells counted. Our impression was, however, that E.C. cells in the experimental groups averaged fewer cytoplasmic granules than E.C. cells studied in the control sections.

Summary. The number of recognizable enterochromaffin cells in the mucosa of the rat duodenum is significantly reduced in response to increased intraluminal pressure. It is suggested that these findings offer histochemical corroboration for the concept that pressure is one mechanism mediating release of 5-hydroxytryptamine from intestinal mucosa.

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1. Erspamer, V., *Pharmacol. Rev.*, 1954, v6, 425.
2. Bertaccini, G., *J. Physiol.*, 1960, v153, 239.
3. Feldberg, W., Toh, C. C., *ibid.*, 1953, v119, 352.
4. Benditt, E. P., Wong, R., *Proc. Inst. Med. Chicago,* 1957, v21, 279.
5. Barter, R., Pearse, A. G. E., *J. Path. Bact.*, 1955, v69, 25.
6. Shepherd, D. M., West, G. B., Erspamer, V., *Nature,* 1953, v172, 357.
7. Bülbring, E., Crema, A., *J. Physiol.*, 1959, v146, 18.
8. Burtner, H. J., Lillie, R. D., *Stain Techn.*, 1949, v24, 225.
9. Bülbring, E., Lin, R. C. Y., *J. Physiol.*, 1958, v140, 381.
10. Pearse, A. G. E., 5-hydroxytryptamine, Edited Lewis, Pergamon Press, London, 1958, p158.

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Increase in Nonspecific Resistance to Infection in Mice Following Administration of Staphylococcal Extracts. (26533)

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The enhanced nonspecific resistance to experimental infections induced by endotoxins derived from Gram-negative bacteria is a well documented phenomenon(1). The biphasic febrile response, Shwartzman reaction, and leucopenia in rabbits produced by these lipopolysaccharides are examples of their other manifold biological activities(2,3). Recently, extracts derived from Gram-positive cocci also have been reported to manifest endotoxin-like properties including the aforementioned and the dermal reactivity of the rabbit to epinephrine(4,5,6).

The experiments described here demonstrate that extracts derived from staphylococci can protect mice against an ordinarily lethal dose of *Escherichia coli,* and substantially increase survival time of mice subjected to lethal doses of *Salmonella typhimurium.* Furthermore, these extracts elicit a pyrogenic response in rabbits; however, the leucopenic response was found to differ qualitatively from that produced by Gram-negative endotoxins.

Materials and methods. Two staphylococcal extracts designated Staphylococcus A lipopolysaccharide control #902761 and control #446423, obtained from Difco Laboratories, are referred to by us as *Staphylococcus* A-1 and *Staphylococcus* A-2, respectively. *S. typhosa* 0-901 lipopolysaccharide (Difco) was used as a representative Gram-negative endotoxin. Mice of the ICR strain (Bellewood Farm), weighing 17 to 18 g, were employed routinely one day after receipt. The culture of *E. coli* was originally obtained from Dr. W. L. Mallmann, Michigan State University, East Lansing. The culture of *S. typhimurium* from our collection was serotyped by the Bureau of Bacteriology, N. J. Dept. of Health. Both cultures were maintained on nutrient agar slants (Difco), transferred to brain heart infusion broth (Difco) and grown at 35°C for 18 hr. The *E. coli* culture was appropriately diluted in sterile broth to give an approximate viable count of 2.1×10^8 cells per ml. Mice received 0.25 ml *i.p.* of this dilution as a challenge inoculum. The 18 hr viable count of *S. typhimurium* approximated 1.3×10^9 organisms per ml. A challenge inoculum of this culture consisted of 0.25 ml *i.p.* of a 10^{-1} or 10^{-3} dilution. Under these conditions, the majority of deaths occurred within 3 days in untreated mice inoculated with *E. coli.* Mice were observed for a period of one week. Depending on the challenge dose of *S. typhimurium,* about half the deaths occurred either by one or 3 days in untreated mice. Animals so infected were observed for 2 weeks.

Pretreatment schedule: Stock solutions of each material were made in saline at 1 mg/ml and kept at –20°C. Mice, in groups of 10, received 1 ml *i.p.* of the endotoxin or staphylococcal extract at 1 or 10 μg/ml, 24 hr prior to infection.

Male rabbits of mixed breed weighing 2 to 2.5 kg were used for determination of febrile and leucopenic responses to *i.v.* pyrogens. Preparation of animals for, and handling during testing were as previously described(7).

Throughout all of this work, rigid precautions were taken to avoid contamination by any extraneous pyrogenic materials. Nonpyrogenic saline was used for control or dilution purposes and all needles, syringes, and glassware were heated at 180°C for 2 to 3 hours.

Results. Preliminary experiments demonstrated that the degree of protection afforded our mice against a challenge of *E. coli* or *S. typhimurium* after the mice were pretreated with *S. typhosa* endotoxin was time and dose dependent as reported by Landy, *et al.*(8). Thus, a model system of endotoxin pretreatment which repeatedly demonstrated significant increased resistance to such infections was chosen to explore the protective effects of extracts of staphylococci.

Both staphylococcal extracts provided at least equivalent protection to a challenge of

TABLE I. Results in Mice Pretreated with Staphylococcal Extracts and *S. typhosa* Endotoxin and Challenged with *E. coli*.

Treatment	Dose, μg, i.p.	No. of mice	% mortality*
Saline	—	30	80
Staph. A-1 extract	10	30	10
S. typhosa endotoxin	10	29	13
Saline	—	20	75
Staph. A-2 extract	10	20	15
S. typhosa endotoxin	10	20	25

* No. of deaths observed 3 days after challenge. $p < .01$.

E. coli as *S. typhosa* endotoxin (Table I).

The data demonstrating increased survival times provided by *Staphylococcus* A-1 and *S. typhosa* endotoxin over the saline controls in infection studies employing *S. typhimurium* are summarized in Table II. With a challenge dose of approximately 1.3 × 10^8 cells per ml, about 60% mortality occurred within 24 hr in the control groups, while the same number of deaths in those groups of mice pretreated with *Staphylococcus* A-1 extract or *S. typhosa* endotoxin occurred by 72 hr. A more sensitive indication of increased resistance to *S. typhimurium* was found when a challenge inoculum of 1.3 × 10^6 cells per ml was employed. While the time to reach about 50% mortality in the saline controls was 3 days, the experimental groups reached this same endpoint at about 8 days. A small percentage of the treated mice still survived at 14 days, but were obviously ill. In other experiments using 1 μg of each material and the larger challenge dose of *S. typhimurium*, increased survival times were evident to about the same extent as shown in Table II.

These infection studies clearly demonstrate significant protecion in mice against *E. coli* and *S. typhimurium* by staphylococcal extracts comparable to that observed with a representative Gram-negative endotoxin.

Both staphylococcal extracts also produced endotoxin-like biphasic fevers, but differed in their effects upon circulating leucocytes and in their relative lack of toxicity as measured by mouse lethality tests. Results of a representative experiment are described in Fig. 1 showing the mean febrile and peripheral leucocyte responses in 5 rabbits given 5 μg of *Staphylococcus* A-1, and for comparison, responses of 3 rabbits injected with an equivalent pyrogenic dose of *S. typhosa* endotoxin (2.5 μg). The early leucopenia produced by the latter is well established and has been described by many workers using preparations derived from a number of genera(2,3,9). The delayed leucopenia in the animals given *Staphylococcus* A-1, without change in num-

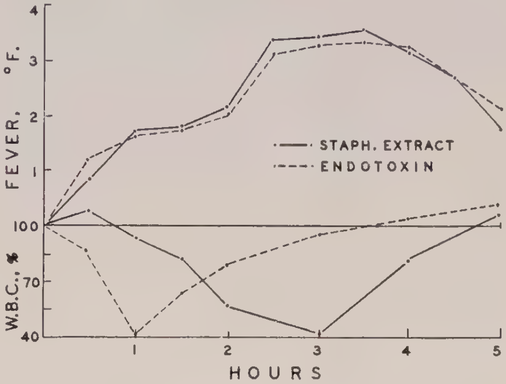

FIG. 1. Differentiation of leucopenic responses to *Staphylococcus* A-1 extract and to *S. typhosa* endotoxin at equivalent febrile reactions.

TABLE II. Results in Mice Pretreated with Staphylococcal Extracts and *S. typhosa* Endotoxin and Challenged with *S. typhimurium*.

Treatment	No. of organisms	No. of mice	% cumulative mortality													
			Days after infection													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
Saline	3.3 × 10^7	30	60	70	83	93	97	97	97	—	—	—	—	—	—	—
Staph. A-1* extract	”	30	3	17	57	77	90	97	97	—	—	—	—	—	—	—
*S. typhosa** endotoxin	”	28	7	11	50	64	71	89	96	—	—	—	—	—	—	—
Saline	3.3 × 10^4	20	0	0	50	85	85	95	95	100	—	—	—	—	—	—
Staph. A-1* extract	”	20	0	0	10	10	25	35	45	50	65	65	70	70	70	70
*S. typhosa** endotoxin	”	20	0	0	5	10	25	45	55	75	80	85	85	85	85	85

* Dose of 10 μg i.p.

bers of leucocytes at 1 hr post-treatment, stands in strong contrast.

Cross-over experiments also demonstrated failure of this extract to elicit the typical 1 hr endotoxin leucopenia. Eleven rabbits were given the extract on day 1 and *S. typhosa* endotoxin on day 2, both at 2.5 μg *i.v.* The mean preinjection and 1 hr postinjection counts were: day 1, 12,320 and 14,850 per mm^3; day 2, 16,840 and 9,800 per mm^3. For 3 rabbits given the pyrogens in reverse order the counts were: day 1, 11,330 and 4,520 per mm^3; day 2, 12,370 and 12,120 per mm^3.

The *Staphylococcus* A-2 extract gave a typical endotoxin-like time course of leucopenia with a maximum drop at 1 hr. However, both extracts differed from Gram-negative endotoxins in that refractoriness to the leucopenic response developed after a first injection. For 8 rabbits given 5 μg of *Staphylococcus* A-2 on day 1, the mean preinjection and 1 hr postinjection leucocyte counts were 10,975 and 6,685 per mm^3. On day 2, 6 of these were given a second equal dose, the counts being 13,540 and 15,720 per mm^3. Two of the 8, and 2 fresh rabbits were given 5 μg of *S. typhosa* endotoxin and for these preinjection and postinjection counts were 11,700 and 6,650 per mm^3, and 10,600 and 6,950 per mm^3, respectively. In other experiments involving daily administration of either *Staphylococcus* extract, counts done on days 4, 5, or 9 revealed neither characteristic time course of leucopenia, unlike results obtained with Gram-negative endotoxins.

When tested for lethality in 24 to 27 g mice, the *Staphylococcus* A-2 extract proved nonlethal at a dose which for *S. typhosa* endotoxin is 100% lethal (Table III); this is in contrast to their equivalent potency as pyrogens.

Discussion. The type and degree of resistance to infection in mice as well as type and degree of febrile response in rabbits produced by the staphylococcal extracts are strikingly parallel to that demonstrated by a representative Gram-negative endotoxin. On the other hand, a leucopenia either of the delayed or 1 hr type is produced by one or the other of the staphylococcal extracts. Over a period of months both materials were tested in several dozen rabbits, comparing each directly or with *S. typhosa* lipopolysaccharide and the peculiar time course of leucopenia has been consistently reproduced. Parenthetically, we are advised by Dr. C. W. Christensen that the Difco Staphylococcus A lipopolysaccharide control #446423 was packaged at a later date from the same bulk of lipopolysaccharides as the control #902761 we originally received. The delayed leucopenia observed cannot be a function of dose, for at lesser doses of endotoxin the early leucopenia still occurs until a dose too small to elicit the response is reached, and at higher doses the leucopenia is simply prolonged from the initial drop. Regarding the prompt leucopenic tolerance to subsequent injections of both staphylococcal extracts, it has been shown that daily injections of endotoxin continue to elicit a leucopenia of constant magnitude(9) and Herion *et al.*(10) have recently extended this describing a decrease in duration but not in degree of leucopenia under similar conditions.

TABLE III. Lethality in Mice of *S. typhosa* Endotoxin and *Staphylococcus* A-2 Extract.

Dose, mg, i.p.	Deaths/Total *S. typhosa* endotoxin	 *Staph.* A-2 extract
.3	4/10	—
.6	10/17	0/10
.9	10/10	0/10

One important consideration is the possibility that these extracts from a Gram-positive organism were contaminated in preparation or subsequent handling during the experimental work by Gram-negative bacteria or their endotoxic components. Several points suggest that this did not occur. First, the effects reported here were all obtained at levels of 10 μg or less, equivalent to a highly potent Gram-negative endotoxin, indicating an initial gross contamination would have been necessary. Secondly, such contamination seems highly unlikely in view of (a), the differentiation in type of leucopenic response discussed above, and (b), the finding that these extracts are nonlethal at corresponding lethal doses of *S. typhosa* endotoxin. Third, Boivin-type preparations from *Brucella abor-*

tus and *Streptococcus* Gr. A. (Difco) were found to be inactive in terms of fever, toxicity, and protection against infection. Fourth, numerous control experiments have demonstrated none of the endotoxic effects, indicating contamination by the diluent, syringes, needles, or glassware was highly improbable. Altogether, these observations strongly suggest that contamination by Gram-negative endotoxins can be excluded.

Several findings of other workers are pertinent to our work. Fruhman has reported mobilization of neutrophils in the peritoneal fluid of the rat and mouse following intraperitoneal injection of a *Staphylococcus* A lipopolysaccharide(11). The directed migration of polymorphonuclear leucocytes and mononuclear phagocytes has been found to reside in a polysaccharide fraction of staphylococci as well as other Gram-positive and Gram-negative organisms(12). A strain of *Alcaligenes* has produced precipitation in agar gel with staphylococcal rabbit antiserum and the erythrocyte sensitizing antigen common to *S. aureus* is apparently present in at least one *Pseudomonas* strain(13). Cross reactivity, then, may be a factor, as well as a peritoneal neutrophilia somewhat akin to the dual mechanism proposed by Rowley for stimulation of nonspecific immunity provoked by endotoxins(14).

Further studies are in progress to implement these preliminary findings and to clarify some of the mechanisms involved.

Summary. Pretreatment of mice by the intraperitoneal route with staphylococcal extracts significantly protects the host against a subsequent lethal challenge of *E. coli* and prolongs survival time of mice infected with *S. typhimurium*. The same extracts were also found to produce a biphasic fever and a leucopenic response in rabbits to which refractoriness was rapidly developed.

The skilled technical assistance of Miriam Graff, Roger Rollins, and Romus Broadway is gratefully acknowledged.

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1. Shilo, M., *Ann. Rev. Microbiol.*, 1959, v13, 255.
2. Atkins, E., *Phys. Rev.*, 1960, v40, 580.
3. Bennett, I. L., Cluff, L. E., *Pharmacol. Rev.*, 1957, v9, 427.
4. Stetson, C. A., *J. Exp. Med.*, 1956, v104, 921.
5. Cremer, N., Watson, D. W., *ibid.*, 1960, v112, 1037.
6. Neter, E., Anzai, H., Gorzynski, E. A., *J. Inf. Dis.*, 1960, v107, 332.
7. Freedman, H. H., *J. Exp. Med.*, 1960, v111, 453.
8. Landy, L., Pillemer, L., *ibid.*, 1956, v104, 383.
9. Freedman, H. H., *ibid.*, 1960, v112, 619.
10. Herion, J. C., Walker, R. I., Palmer, J. G., *Am. J. Phys.*, 1960, v199, 809.
11. Fruhman, G. J., PROC. SOC. EXP. BIOL. AND MED., 1959, v102, 423.
12. Suter, E., *Bact. Rev.*, 1956, v20, 94.
13. Oeding, P., *ibid.*, 1960, v24, 374.
14. Rowley, D., *J. Exp. Med.*, 1960, 111, 137.

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Immunologic Identification of Coxsackie A21 Virus with Coe Virus.* (26534)

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By means of reciprocal neutralization tests, the recently described Coxsackie group A, type 21 virus prototype strain was found to be immunologically identical to the Coe virus, recovered from individuals with mild respiratory illness.

The Coe virus, described by Lennette *et al.* (1), was isolated from the throat washings of 4 military recruits with mild respiratory illness. Subsequently, isolations of the Coe virus were reported from patients with respiratory illness in Great Britain(2), the Netherlands(3), and the Washington, D.C. area(4), as well as in a recent outbreak of respiratory disease in the same military installation from which the virus was originally isolated(5). The agent has been further implicated in the etiology of respiratory disease by the recent demonstration of Parsons *et al.*(6) that human volunteers inoculated with the virus developed illnesses resembling the common cold.

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Pereira and Pereira(2) found the Coe virus to be ether resistant, and their studies, as well as recent investigations in this laboratory(7), have indicated that the size of the Coe virus approximates that of the enteroviruses. The cardinal characteristic of the agent which distinguishes it from most of the other members of the enterovirus group is its inability to produce a cytopathic effect in monkey kidney cell cultures; however, the agent produces degeneration similar to that of the enteroviruses in a variety of mammalian cells in continuous passage as well as primary cultures of human tissues(1,2).

Attempts were made in this laboratory to propagate all 4 of the originally described Coe virus strains, as well as a recently isolated strain, in suckling mice, and although the inoculated animals occasionally showed transitory illness, it was impossible to establish the virus in infant mice even through multiple, blind passages of brain and muscle material (1). This was also the experience of Pereira and Pereira(2) employing a strain of the virus isolated in Great Britain; not only was it impossible to propagate the virus strain in suckling mice, but these workers were unable to demonstrate virus in the tissues of inoculated mice which had displayed evidence of illness.

The prototype strain (Kuykendall strain) of Coxsackie group A, type 21 virus was also originally isolated in this laboratory. The isolation was made in tissue cultures of human embryonic skin and muscle, the virus being recovered from the stool of an individual with paralytic poliomyelitis whose stools also yielded a type 1 poliovirus. The fourth tissue culture passage material was inoculated into 12 suckling mice, 2 of which became moribund at 10 days. Brain and muscle tissue from these ill mice was passaged into additional suckling mice and, while the brain tissue produced no evidence of illness, 7 of the 12 mice inoculated with the muscle tissue suspension became ill at 8-9 days and the muscles showed gross lesions. Muscle tissue from these mice failed to produce symptoms in 2 litters of mice, but 2 additional attempts were made to infect mice with the same material, and both were successful. At the fourth passage level of infected mouse muscle, most of the inoculated mice became paralyzed at 4-6 days, and the strain was well established in suckling mice at the 5th passage, producing paralysis at 5 days after inoculation. The 7th mouse passage material was sent to the laboratory of Dr. Gilbert Dalldorf where the virus strain was designated as the prototype of Coxsackie group A, type 21 on the basis that in newborn mice it induced flaccid paralysis with marked myositis, but no lesions in the central nervous system(8). Interestingly, it was also found by Dr. Dalldorf's laboratory that the strain occasionally caused paralysis in 10-12 g mice, but could not be propagated in these older mice. The virus was shown to be non-cytopathogenic in monkey kidney cell cultures, but cytopathogenic in HeLa cell cultures and human amnion and uterine tissue cultures(8).

When the Coe virus was recently examined in neutralization tests against immune sera to the newly-recognized group A Coxsackie virus types 20-24, it was found that the virus was neutralized by immune serum to Coxsackie A21 virus. The results of cross-neutralization tests with the viruses and hamster immune sera to Coe and Coxsackie A21 (Kuykendall) strains are presented in Table I. In monolayer and colorimetric neutralization tests(1) both immune sera displayed essentially the same endpoints against both viruses, indicating that immunologically the 2 strains are very closely related, if not identical.

The demonstration of the immunologic identity of these 2 viruses further illustrates that the pathogeniciy of an enterovirus strain for suckling mice is not a valid criterion for placing the immunotype in the Coxsackie group. It is now well known that certain strains of ECHO virus types 9 and 10 (reo-

TABLE I. Results of Reciprocal Neutralization Tests with Coe and Coxsackie Group A, Type 21, Viruses and Immune Sera.

Test system	No. viral TCD_{50} in test	Neutralization titers of serum: Coe immune serum vs Coe virus	Coe immune serum vs Coxsackie A21 virus	Coxsackie A21 immune serum vs Coe virus	Coxsackie A21 immune serum vs Coxsackie A21 virus
Monolayer cell cultures in tubes	1000	1024*	—	128	—
	300	1024	512	128	128
	100	1024	1024	256	256
	30	1024	1024	512	512
	10	—	2048	—	512
Metabolic inhibition (colorimetric)	560	2048	—	256	—
	180	1024	2048	512	512
	56	4096	2048	512	1024
	18	≥ 4096	≥ 4096	512	1024

* Reciprocal of highest dilution of serum neutralizing test dose of virus.

virus) are pathogenic for infant mice, while other immunologically identical strains do not propagate in infant mice(9), and strains of Coxsackie viruses, particularly A9, have been recognized which are not pathogenic for suckling mice(10,11). In the case of Coe and Coxsackie A21 viruses, it would appear that the majority of strains of the immunotype thus far described lack pathogenicity for infant mice, and the Kuykendall strain may conceivably represent an aberrant strain of the Coe virus. (The pathogenicity of the Coxsackie A21 virus in young adult mice is much the same as that of Coe virus in suckling mice in that illness is occasionally induced, but the strain cannot be propagated.) Reclassification of the enteroviruses as numerical types on the basis of immunologic distinctness rather than biological properties (pathogenicity) should prevent further confusion in classification arising from differences in biological properties of various strains of an immunotype.

Of particular interest is the fact that an enteric virus immunotype, as represented by a strain possessing certain "Coxsackie" characteristics, has been implicated as the etiologic agent of respiratory illness with cold-like symptoms. This further extends the spectrum of clinical manifestations attributable to viral strains immunologically identical to "Coxsackie" viruses.

Summary. Cross neutralization tests in HeLa cell cultures, using both the monolayer and metabolic-inhibition technics, show the Coe virus, etiologically associated with respiratory disease, to be immunologically indistinguishable from the recently-described Coxsackie group A, type 21, virus.

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1. Lennette, E. H., Fox, V. L., Schmidt, N. J., Culver, J. O., *Am. J. Hyg.*, 1958, v68, 272.
2. Pereira, M. S., Pereira, H. G., *Lancet*, 1959, v2, 539.
3. van der Veen, J., Dei, K. G., Prins, A., *Ned. T. Geneesh.*, 1960, v104, 617.
4. Johnson, K., Personal communication.
5. Lennette, E. H., Stallones, R. A., Beach, D. J., to be published.
6. Parsons, R., Bynoe, M. L., Pereira, M. S., Tyrrell, D. A. J., *Brit. Med. J.*, 1960, 1776.
7. Frommhagen, L. H., to be published.
8. Sickles, G. M., Mutterer, M., Plager, H., Proc. Soc. Exp. Biol. and Med., 1959, v102, 742.
9. Committee on the Enteroviruses., *Am. J. Pub. Hlth.*, 1957, v47, 1556.
10. Habel, K., Silverberg, R. J., Shelokov, A., *Ann. N. Y. Acad. Sci.*, 1957, v67, 223.
11. Kalter, S. S., Hall, A. D., *Proc. Soc. Am. Bact.*, 1959, 60.

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Stimulation of Pancreatic Islet Cells by 2-Deoxy-D-Glucose.* (26535)

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2-Deoxy-D-Glucose (2-DG) inhibits normal utilization of glucose in the cell and leads to cellular glucopenia(1). In intact animals this condition is accompanied by marked adrenaline release which seems to play a major role in induction of hyperglycemia by 2-DG(2). The present study investigated whether 2-DG affects the alpha and beta cells of the pancreatic islets, the functions of which are intimately related to glucose metabolism.

Material and methods. The experiments involved white inbred male rats, 175-275 g, maintained on standard diet. During acute experiments no food was given, but free access to water was allowed. 2-DG† was dissolved in water, 50 mg/ml, and administered subcutaneously. In acute experiments the single injection (50 mg/100 g b.w.) was given at 8 a.m. In long term experiments the same dose was injected at 8 a.m. and 7 p.m. for 6 days. Control animals received a corresponding quantity of distilled water. In acute experiments glucose in tail blood was determined at different times following 2-DG, in long term experiments only prior to and one hour after the first injection on days 1 and 6. Glucose was determined colorimetrically using condensation with *o*-toluidine(3), a method in which 2-DG is only one-tenth as chromogenic as glucose(2). The animals were killed by decapitation at various times after 2-DG, the experiments being arranged so that all rats were killed about 8:00 p.m.

Pancreatic islet cell activity was evaluated on basis of changes in stainability, granulation and nuclear size. The pancreas was fixed in Bouin's fluid and stained with chromhaematoxylin-Ponceaufuchsin or with paraldehydefuchsin-Ponceaufuchsin. Nuclear size was determined with a planimeter using section 7 μ thick; about 50-70 alpha and 50-70 beta nuclei were traced off at a magnification of approximately 2,000. Under these conditions the standard error of a single determination in percentage of ordinary mean values is 2.1-3.0 for alpha cells and 1.1-1.5 for beta cells (4).

Results. Pronounced hyperglycemia appeared as early as 30 minutes after administration of 2-DG (50 mg/100 g b.w.) and lasted for at least another 4-6 hours. Blood sugar approached control values at 12 hours (Table I). During this period marked cytological changes appeared in beta and alpha cells. There was enlargement of beta cell nuclei, highly significant 6 hours after 2-DG and probably significant after 9 hours (Table I). Concomitantly stainability and granulation of the cytoplasm decreased (Fig. 1 and 2), particularly in the central parts of the islets. After 12 hours all these parameters had become normal.

In the alpha cells cytological changes appeared analogous to those described for the beta cells. Although not statistically significant until 6 hours after injection of 2-DG, the alpha cell nuclei seemed enlarged after 1-3 hours; at 9 hours nuclear size was still increased (probably significant) while at 12 hours no difference could be demonstrated as compared to controls (Table I). The decreased stainability and degranulation of the cytoplasm of the alpha cells is shown in Fig. 3 and 4. In those alpha cells presenting the most marked nuclear changes, vacuolation was visible. Furthermore, the nuclear bodies stained intensely in 2-DG-treated animals.

In long term experiments blood sugar rose from 81 ± 2.7 mg% to 207 ± 11.4 one hour after first injection on day 1, the corresponding figures for day 6 being 78 ± 2.1 and 160 ± 9.2. The average size of the beta cell nuclei was 29.6 μ^2 in treated rats, as com-

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* Supported by grants from the Åhlén Foundation and Swedish Medical Research Council.

† Obtained from Aldrich Chemical Co., Milwaukee, Wisc., courtesy Cancer Chemotherapy, Nat. Service Center, NIH, Bethesda.

TABLE I. Blood Glucose and Nuclear Area of Alpha and Beta Cells Following a Single Injection of 2-DG.

Time after inj., hr	Blood glucose, mg % ± S.E. Controls	2-DG, 50 mg/100 g b.w.	Alpha cells Mean value, μ^2	P	Beta cells Mean value, μ^2	P
0	88 ± 1.7	84 ± 2.6	22.0*		25.5*	
½	—	200 ± 6.6				
1	92 ± 3.9	271 ± 8.0	23.3	>.05	26.1	>.05
2	90 ± 3.4	242 ± 6.2				
3	91 ± 3.7	—	23.7	>.05	25.8	>.1
4	88 ± 1.5	195 ± 8.1				
6	90 ± 4.0	122 ± 5.1	25.2	<.01	27.8	<.001
9	91 ± 4.1	110 ± 3.5	24.1	<.02	29.2	<.05
12	92 ± 4.6	103 ± 4.1	22.3	>.1	25.7	>.1

* Non-inj. controls.

P = probability.

Fig. 1

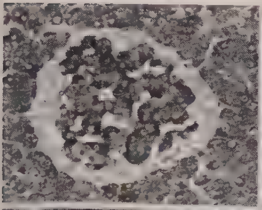
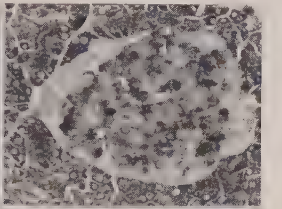


Fig. 2



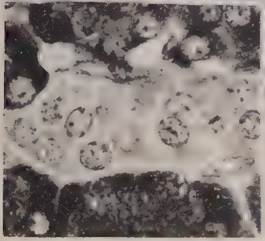


Fig. 3

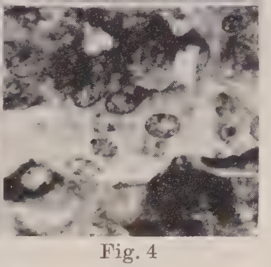


Fig. 4

FIG. 1. Islet in a control rat. Paraldehydefuchsin-Ponceaufuchsin. × 180.

FIG. 2. Islet 9 hr after 2-DG. Reduced stainability in beta cells most pronounced in central parts of islet. Same stain and magnification as in Fig. 1.

FIG. 3. Group of alpha cells in an islet of a control rat. Same stain as in Fig. 1. × 600.

FIG. 4. Group of alpha cells showing reduced stainability and granulation with vacuolisation following 2-DG. Same stain and magnification as in Fig. 3.

pared to 26.0 in controls (P = <0.01). For the alpha cells the corresponding values were 24.6 μ^2 and 22.3 μ^2 respectively (P = 0.01). By mistake 2 control rats were given 2-DG twice on the day prior to termination of the experiment, possibly causing some enlargement of the pancreatic islet cell nuclei in the control group. Signs of degeneration of alpha or beta cells were not noted, either in acute or in long term experiments. Increased mitotic activity was not observed.

Discussion. The combination of nuclear enlargement, degranulation, and decreased stainability of the cytoplasm in the beta cells of the pancreatic islets probably represents increased activity(5). While increased activity of the beta cells is generally considered to result in insulin secretion, the interpretation of similar changes in the alpha cells is disputed. It seems likely that these changes in the alpha cells also reflect stimulation, and there are reasons to believe that alpha cells produce glucagon(6,7). Our findings may then indicate that 2-DG may cause increased secretion of both insulin and glucagon.

With respect to the beta cells, there is general agreement that hyperglycemia stimulates insulin secretion, and it seems likely that this was the effective stimulus in our experiments. On the other hand, insulin produced hypoglycemia stimulates the alpha cells(8), and it seems paradoxical to find evidence of alpha cell stimulation in the presence of hyperglycemia. However, 2-DG imposes cellular glucopenia and it is reasonable to assume that this is the effective stimulus for the alpha cells. It may be that an identical mechanism is involved in alpha cell stimulation following both insulin and 2-DG. Similarly, the centrally located receptor system regulating adrenaline secretion has been found to be stimulated by either insulin or 2-DG.

Summary. In the intact rat 2-Deoxy-D-Glucose (2-DG) leads to decreased stainability and degranulation of the cytoplasm and increased nuclear size of both alpha and beta cells of the pancreatic islets. Maximal changes occurred 6 hours after administration of 2-

DG. The findings are interpreted to indicate increased activity in both types of cells and the underlying regulatory mechanisms are discussed.

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1. Landau, B. R., Lubs, H. A., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 124.

2. Hökfelt, B., Bygdeman, S., *ibid.*, in press.

3. Hultman, E., *Nature*, 1959, v183, 108.

4. Hultquist, G., *Acta Pathol. et Microbiol. Scand.*, 1959, v47, 153.

5. Volk, B. W., Lazarus, S. S., *Diabetes*, 1960, v9, 264.

6. Logothetopoulos, J., Sharma, B. B., Salter, J. M., Best, C. H., *ibid.*, 1960, v9, 278.

7. Unger, R. H., Eisentraut, A. M., McCall, M. S., Madison, L. L., *J. Clin. Invest.*, 1960, v39, 1036.

8. Hökfelt, B., Hultquist, G., *Acta Physiol. Scand.*, 1958, v43, 8.

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Differential Anaphylactic Susceptibility of Chicken and Guinea Pig Intestine.* (26536)

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The possibility of using the intestine of chickens, instead of that of guinea pigs, in the Schultz-Dale reaction was investigated. Since we were unable passively to sensitize the chicken intestine, a study was made of factors that might contribute to the differential anaphylactic susceptibilities of chickens and guinea pigs.

Materials and methods. White Leghorn chickens, weighing 1.3 to 1.7 kg, were given a single intravenous sensitizing injection of chicken or rabbit antiserum containing 25 mg of antibovine serum albumin (anti-BSA) antibody per kg body weight. Both antiserums contained 1.5 mg of precipitating antibody protein per ml of serum as estimated by the quantitative precipitin test (8% salt concentration used for chicken serums)(1). Chickens were fasted for 16 to 18 hours, killed by a blow on the head, and 15 cm of the lower portion of the small intestine cranial and adjacent to the caecum was carefully removed and kept moistened at all times with modified Tyrode solution(2). The intestinal contents were carefully removed by flushing the intestine with 20 ml of fresh Tyrode solution. For each experiment, one control and one experimental strip, 4 cm in length, were immersed in a 120 ml muscle bath of the Schultz-Dale apparatus(3) containing Tyrode solution at 40°C and a stream of O^2 (90%) plus CO_2 (10%) was used to aerate and stir the bath. The intestinal strips were allowed to equilibrate in the bath for 15 minutes, then their reactions were tested by adding 9 mg of BSA, 25 to 800 μg (in 2-fold increments) of histamine, and 0.1 to 0.8 μg (in 2-fold increments) of acetylcholine chloride. After each test the bath was drained, the intestinal strips washed, and fresh Tyrode solution added and equilibrated for 8 minutes.

Essentially the same procedure was followed for the guinea pig. Guinea pigs weighing 0.4 to 1 kg were divided into 5 groups. The first 2 groups received 0.25 and 0.5 mg of rabbit anti-BSA serum per kg body weight, respectively, and the third group received 25 mg of chicken anti-BSA serum per kg body weight. The other 2 groups, which served as controls, were injected with normal rabbit and chicken serums, respectively. The contractility of the ileum, 4 cm in length, was tested with 9 mg of BSA, 0.025 to 0.2 μg (in 2-fold increments) of histamine, and 0.01 to 0.08 μg (in 2-fold increments of acetylcholine in the Schultz-Dale bath maintained at 38°C.

Results. As indicated in Table I, 25 mg of either rabbit or chicken antibody per kg

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‡ Contribution No. 2647.

TABLE I. Schultz-Dale Reactions of Passively Sensitized Guinea Pig and Chicken Intestine.

	Sensitizing antiserum	Antibody inj., mg/kg body wt	Response to BSA, No. animals positive/No. tested	Minimum dose required for contraction in 120 ml bath	
				Histamine, μg	Acetylcholine, μg
Guinea pig intestine	Rabbit anti-BSA	.25	0/2	.1, .1	.04, .04
		.5	3/3	.1, .1, .2	.04, .04, .08
	Control*		0/2	.1, .1	.04, .04
	Chicken anti-BSA	25	0/3	.05, .1, .1	.02, .04, .04
			0/2	.05, .1	.04, .08
			Avg	.1	.04
Chicken intestine	Rabbit anti-BSA	25	0/3	100, 400, 400	.4, .4, .4
	Control*		0/2	50, 100	.1, .4
	Chicken anti-BSA	25	0/3	50, 200, 800	.2, .8, .4
	Control		0/2	100, 100	.4, .8
			Avg	200	.4

* Controls received equivalent amounts of normal serum.

body weight failed to sensitize the chicken intestine. Attempts to sensitize the guinea pig intestine with 25 mg of chicken antibody per kg body weight also failed. On the other hand the guinea pig intestine was successfully sensitized with as little as 0.5 mg of rabbit antibody per kg body weight.

Guinea pig intestine contracted with an average of 0.1 μg of histamine, whereas chicken intestine required an average of 200 μg. The intestinal strip of the guinea pig also showed higher sensitivity to acetylcholine than did that of the chicken: an average of 0.04 in guinea pigs *vs.* an average of 0.4 μg in chickens.

Discussion. Early in this century, it was reported that the chicken antiserum lacked the "necrotizing effect" on guinea pig tissues which the rabbit antiserum possessed(4), and that rabbit anti-sheep serum did not passively sensitize chickens(5). Recently Ovary(6) found that chicken anti-rabbit gamma globulin serum would not sensitize the guinea pig for passive cutaneous anaphylaxis (PCA) test but that the reverse PCA test would succeed using chicken antibody and rabbit gamma globulin as an antigen, which, according to the author, can be "fixed" by guinea pig tissues.

It appears from the above that the failure of the chicken antibody to sensitize the guinea pig intestine in our experiment may be due to a lack of affinity between chicken antibody and guinea pig intestine. The inability of rabbit or chicken antibody to sensitize chicken intestine can also be explained similarly. However, there are other factors to be considered; Ishizaka, Ishizaka, and Campbell (7) have shown that toxicity of the antigen antibody complex is dependent upon ratio of the antigen to antibody. Accordingly, it may be that the negative cases reported here may be due to failure of the complexes to assume the proper antigen to antibody ratio. The same authors(8) reported that soluble complexes composed of chicken antibody and rabbit gamma globulin did not confer cutaneous anaphylaxis in the guinea pig. However complexes formed *in vivo* may be of a different nature than those formed *in vitro*, as indicated by the apparent contradictory results obtained by Ovary and by Ishizaka *et al.*

The low histamine and acetylcholine sensitivity of the chicken intestine suggests that even if the toxic complex did form, the chicken intestine may not contract due to insufficient amounts of mediators liberated. Makinodan *et al.*(9) reported that uniform fatal anaphylactic shock did not occur in actively sensitized chickens unless the circulating precipitin titer was greater than 7 mg§

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§ The original data given were 0.75 mg antibody N/ml as determined in 1% saline. This was multiplied by a factor of 1.5 since antibody content increases by the factor when determined in 8% saline (1).

anti-BSA protein per ml serum. The total amount of circulating antibody would have been about 420 mg in a one-kg chicken. To study passive anaphylaxis, one would have to inject several times this amount to secure an antibody titer of 7 mg antibody/ml serum one or 2 days after injection. Guinea pigs can be passively sensitized, with fatal anaphylaxis, with 0.75 mg of antibody per kg body weight, and circulating antibodies are no longer detected when challenged 2 days later(10). From the above comparison, the difference in susceptibility between guinea pig and chicken to passive sensitization and the difference in histamine sensitivity of the 2 animals appear to be of similar magnitude. Further study on the passive anaphylaxis of the 2 animals is desirable to clarify this point.

Summary. Tested by the Schultz-Dale Technic, chicken antibody failed to sensitize the chicken or the guinea pig intestine. Rabbit antibody sensitized guinea pig intestine but failed to sensitize chicken intestine. The minimum doses of histamine and acetylcholine required for contraction of the chicken intestine were considerably larger than those required for guinea pig intestine.

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1. Goodman, M., Wolfe, H. R., Norton, S., *J. Immunol.*, 1951, v66, 225.
2. Feigen, G. A., Campbell, D. H., *Am. J. Physiol.*, 1946, v145, 676.
3. Campbell, D. H., McCasland, G. E., *J. Immunol.*, 1944, v49, 315.
4. Uhlenhuth, P., Haendel, Z., *Z. Immunitatsforsch.*, 1909, v3, 284.
5. Friedberger, E., Hartoch, O., *ibid.*, 1909, v3, 581.
6. Ovary, Z., *Immunology*, 1960, v3, 19.
7. Ishizaka, K., Ishizaka, T., Campbell, D. H., *J. Exp. Med.*, 1959, v109, 127.
8. ———, *J. Immunol.*, 1959, v83, 105.
9. Makinodan, T., Wolfe, H. R., Goodman, M., Ruth, R., *ibid.*, 1952, v68, 219.
10. Kabat, E. A., Landow, H., *ibid.*, 1942, v44, 69.

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Effect of 6-Mercaptopurine on Homograft Reaction in Rats. (26537)

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Attempts at modification of the immune response to skin homografts by means of physical agents such as x-irradiation, cortisone and nitrogen mustard have been relatively unsuccessful. Recently, Schwartz and his associates(1-3) suppressed the immune response to soluble foreign protein in rabbits with 3 to 6 mg/kg/day of the antimetabolite 6-mercaptopurine (6-MP). Meeker and his associates(4) reported a significant increase in skin homograft survival in rabbits by using 6 to 12 mg/kg/day of 6-MP administered for relatively short periods. However, Hubay *et al.*(5), using doses up to 24 mg/kg/day in rats for short periods of time, found no prolongation of homograft survival. The present study was designed to evaluate further the effect of 6-MP on the homograft reaction in rats by increasing the dosage to the maximum tolerated level and by long term administration of the drug prior to grafting.

Method. Skin homograft survival was studied in 241 young male rats of the Long-Evans strain which were given freshly prepared 6-MP* intraperitoneally. Twenty additional rats were used as controls. The injection period before homografting varied between 0 and 174 days (Table 1). The drug was administered after grafting until the homograft had been completely destroyed. The rats were divided into 12 groups and were given 6-MP in dosages ranging from 20 to

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* 6-Mercaptopurine was kindly supplied by Dr. Donald S. Searle, Burroughs-Wellcome and Co., Inc.

TABLE I. 6-Mercaptopurine Dosage and Homograft Survival in Young Male Rats.

Drug dosage (3× per wk), mg/kg	No. of rats	Inj. period in days before grafting	Graft survival in days
Controls	20		11 (10–16)*
20	24	16 (10– 18)*	11 (10–15)
30	42	121 (119–125)	12 (10–16)
40	39	140 (88–174)	12 (10–16)
60	14	29 —	12 (10–16)
80	14	30 —	13 (11–17)
100	14	17 —	12 (10–16)
150	16	10 —	16 (11–39)
200	16	10 —	15 (11–23)
250	31	5 (3–6)	16 (11–46)
300	15	4 —	15 (11–25)
350	16	0 0	— —

* Mean and (range).

350 mg/kg 3 times per week. Full thickness, suprapannicular skin grafts, 1.2 cm in diameter, were taken with a skin punch especially adapted for rats(6). Duration of skin homograft survival was determined by its gross and microscopic appearance. Onset of the homograft reaction was detectable by the appearance of edema, slight dessication with brownish discoloration and diminution of graft size. Shrinkage of the graft without edema or dessication was usually associated with mild reactions and required several days for completion.

Results. The longest mean prolongation of homograft survival beyond that of the control grafts was only 5 days. Neither long term administration nor high doses of 6-MP were effective in suppressing the homograft reaction. Rats are remarkably tolerant to 6-MP given for long periods or in high dosages. To exceed this tolerance required a progressive increase in dosage of 6-MP to 350 mg/kg 3 times a week. At this dosage mean survival of the animals was only 5 days, less than the 11-day survival of the control grafts. The animals receiving 300 mg/kg of 6-MP 3 times per week survived long enough to permit evaluation of the homografts (Table I).

Conclusion and summary. There was an apparent, brief prolongation of the mean skin homograft survival in 241 rats which were given 6-MP. However, neither long term administration nor high doses of this drug consistently prolonged survival time. A few of the treated animals in the higher dosage groups had unusually long graft survivals which accounted for the over-all increase of mean graft survival. Most of the animals in all groups sloughed off their grafts at or near the expected time.

1. Schwartz, R., Stack, J., Dameshek, W., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 164.

2. Schwartz, R., Dameshek, W., *Nature*, 1959, v183, 1682.

3. Schwartz, R., Eisner, A., Dameshek, W., *J. Clin. Invest.*, 1959, v38, 1394.

4. Meeker, W., Condie, R., Weiner, D., Varco, R. L., Good, R. A., PROC. SOC. EXP. BIOL. AND MED., 1959, v102, 459.

5. Hubay, C. A., Powell, A., Holden, W. W., *S. Forum*, 1960, v11, 468.

6. Gottfried, B., Padnos, M., *Transpl. Bull.*, 1959, v6, 427.

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Synthesis and Electrophoretic Distribution of Antibodies in the Amphibian, *Bufo Marinus.** (26538)

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In spite of considerable research on tissue transplantation in the amphibia(1), there is relatively little published information on agglutinating and precipitating antibody in this class or in fishes and reptiles. From a phylogenetic standpoint this is unfortunate because a comparative study might yield much information. Recent work(2) has shown that the desert lizard, *Dipsosaurus dorsalis* can synthesize agglutinating antibodies for *Salmon-*

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ella typhosa and Bisset(3-5) and Cushing(6) demonstrated that frogs and fish can produce agglutinins. Since the lower vertebrates are poikilothermic, they need to be maintained within the temperature range at which they are normally active, to obtain optimal antibody formation. In Bisset's work, for instance, frogs produced antibody when kept at 20°C but not at 8°C.

Although there have been several reports on electrophoretic separation of serum proteins of the lower vertebrate classes(7-15) there is need for further studies. Some of the serum patterns described appear to differ from the normal electrophoretic distribution of serum proteins in man and other mammals. These differences may prove to have phylogenetic significance. It will also be important to determine the location of antibodies in the serum fractions of the lower vertebrates.

In our investigation, we found that the toad *Bufo marinus* would synthesize precipitating antibody to rabbit gamma globulin and agglutinating antibody to *Salmonella typhosa* after a relatively short period of immunization. Details of the methods for production and measurement of antibodies together with a study of the electrophoretic distribution of agglutinins in 10 different antiserums are reported.

Materials and methods. To prepare the antigen used for immunization, a solution of rabbit gamma globulin (RGG) was mixed with *Salmonella typhosa* H vaccine. The RGG (Pentex Co.) was dissolved in saline (20 mg/ml) and the *S. typhosa* H antigen(2) diluted to a density of 10% transmittance at 540 mμ. Shortly before use the two were mixed in equal proportions.

On the first day of immunization, 10 large (3½ inch to 5 inch) adult *Bufo marinus* were injected with 0.2 ml of antigen. On the second and third days, they received 0.6 ml. After a 4-day rest period, 3 more injections of 0.6 ml each were given on consecutive days. All injections were made with a 23 gauge needle into the subcutaneous space of the sacral region. These animals plus 10 additional non-immunized controls were maintained in covered jars containing a layer of damp sphagnum moss. Ambient temperature was maintained at 25°C ± 1°. All animals were fed, *ad lib.*, on Tenebrio larvae. Seven days after the last injection the animals were bled by cardiac puncture and the blood allowed to clot. The serum was harvested and used to determine the titer of agglutinating antibody for *S. typhosa* H antigen as in(2). Precipitins were measured by layering various dilutions of the RGG antigen over undiluted antiserum in capillary tubes.

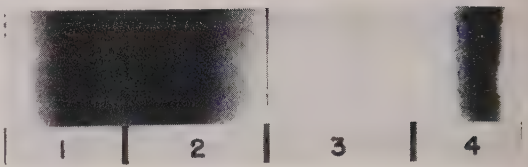

FIG. 1. Paper electrophoresis strip stained with bromphenol blue to show the 4 major fractions of serum from *B. marinus*. Point of origin was near center of fraction 1.

To determine which electrophoretic fraction contained the antibody, the serums were analyzed in a Beckman Spinco electrophoresis cell with a Duostat power supply and B-2 veronal buffer, pH 8.6. Amperage was held constant at 2.5 milliamperes (8 strips). After preliminary determinations had disclosed that there were 4 well-defined components in the serum of *B. marinus* (Fig. 1), each antiserum was fractionated in the following way:

Each of 7 filter paper strips (Spinco no. 300-028) was spotted with 40 μl of serum (4 × 10 μl) and placed in the Spinco cell. Strip no. 8 was also inserted, but was left blank since the serum fractions tended to migrate more slowly on this strip. After 24 to 32 hours of migration, the strips were removed and one strip was quickly dried with a hair dryer and rapidly transferred through methanol, bromphenol blue solution and 5% acetic acid to develop the protein bands. While still wet, the remaining 6 strips were cut along lines between each fraction (Fig. 1) using the stained strip as a guide. The 6 pieces of filter paper from Fraction no. 1 were placed in a 40 ml centrifuge tube and macerated in 15 ml of saline solution at 37°C. This process was repeated with each of the other fractions. After 30 minutes incubation, the tubes were centrifuged, the supernate was

TABLE I. Results of Precipitation and Agglutination Tests on Serums of 10 Non-immunized and 10 Immunized *Bufo marinus.*

No. of animals with:	Group (10 animals each) Immunized	Non-immunized
Precipitating antibody (RGG)	10/10*	0/10
Agglutinating antibody (Typhoid H)	10/10	1/10
Titers: 1:10	0/10	1/10
1:320	1/10	0/10
1:640	4/10	0/10
1:1280	5/10	0/10

* No. positive/total.

saved and the pulp again rinsed with 15 ml of saline. The rinsing was repeated with 10 ml of saline, and the 3 eluates combined to give a total volume of 40 ml for each fraction. This eluate was dialyzed for 24 hours against 0.1% sodium chloride, then reduced to 1/10 volume by perevaporation before an electric fan at room temperature. This fluid was adjusted to 1:20 in terms of the 240 μl of serum applied to the 6 strips and used to set up agglutination tests as in(2). When precipitin tests with RGG were found to be negative at a 1:20 dilution of serum, the fractions were again concentrated by a similar procedure to equal a dilution of 1:2 in terms of the whole serum.

Results. Agglutination tests with *S. typhosa* H antigen failed to reveal any agglutinins in the non-immunized group except for one animal which had a titer of 1:10. None of these serums precipitated with RGG (Table I).

In the immunized group, *S. typhosa* H titers ranged from 1:320 to 1:1280 and all serums produced positive ring precipitin tests with RGG. Although not shown in the table, positive ring tests were obtained with the following concentrations of RGG: 10, 1, and 0.1 mg/ml. Agglutination tests were read after 2 hours incubation in a 37°C water bath and ring tests after 2 hours at room temperature.

When the 4 major electrophoretic fractions (Fig. 1) were eluted from paper strips, the dilution of each in terms of original serum was 1:20. When this was diluted with an equal volume of H antigen in the agglutination test, the dilution was 1:40. All fractions except no. 1 yielded titers of less than 1:40, but fraction no. 1 from each serum agglutinated strongly with titers ranging from 1:160 to 1:640 (Table II). In the case of 2 serums these titers equalled those of the unfractionated serum and in the others were one or 2 tubes lower. When serum from non-immunized animals was separated electrophoretically and eluted by the same process, no agglutinins were found in any fraction.

Despite repeated attempts to demonstrate precipitating antibody in one of the 4 fractions, results were negative even when the serum was concentrated to equal a dilution of 1:2 of the original serum. Further work has been hampered by the small volume of serum available from each animal, but additional studies are contemplated using immunoelectrophoresis and agglutination of RGG-sensitized erythrocytes.

Discussion. Although there is relatively little experimental work on comparative immunology of the lower vertebrate classes the available data suggest that reptiles, amphibia and fishes can form antibodies of the sort that have been widely investigated in mammals and certain birds. The results presented here are in accord with this viewpoint. *B. marinus* was shown to produce typhoid H agglutinins as well as precipitating antibody for rabbit γ-globulin, and the agglutinin was found in the slowest-moving of the 4 major serum components. On the basis of its electrophoretic mobility, this component would be consid-

TABLE II. Reciprocals of *S. typhosa* H Agglutinin Titers with Eluates from Paper Electrophoresis Strips.

Animal No.	Titer of whole serum	Major electrophoretic fraction 1	2	3	4
21	640	320*	<40	<40	<40
22	1280	640	<40	<40	<40
23	1280	640	<40	<40	<40
24	1280	320	<40	<40	<40
25	1280	320	<40	<40	<40
26	640	640	<40	<40	<40
27	320	320	<40	<40	<40
28	640	320	<40	<40	<40
29	640	160	<40	<40	<40
30	1280	640	<40	<40	<40

* Dilutions are expressed in terms of volume of whole serum applied to strips.

ered analogous to the gamma globulin fraction of mammals.

Additional work is required to assess the full phylogenetic significance of these results. It will be of interest to ascertain whether a similar electrophoretic distribution of antibodies is found in the serum of other lower vertebrates and in animals immunized to antigens other than those used here.

Summary. Adult specimens of *B. marinus* maintained at 25°C were immunized with a mixture of *S. typhosa* H antigen and rabbit gamma globulin (RGG). Each animal produced precipitins for the RGG and agglutinins for typhoid H after a short period of immunization. When the antiserums were separated electrophoretically, the agglutinins were found in the slowest-moving of the 4 major serum components. On the basis of electrophoretic mobility, this fraction is analogous to the gamma globulin of mammals.

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1. Cushing, J. E., Campbell, D. H., *Principles of Immunology,* 1957, McGraw Hill, N. Y.

2. Evans, E. E., Cowles, R. B., Proc. Soc. Exp. Biol. and Med., 1959, v101, 482.

3. Bisset, K. A., *J. Path. Bact.,* 1947, v59, 301.

4. ———, *ibid.,* 1947, v59, 679.

5. ———, *ibid.,* 1948, v60, 87.

6. Cushing, J. E., *J. Immunol.,* 1942, v45, 123,

7. Deutsch, H. F., McShan, W. H., *J. Biol. Chem.,* 1949, v180, 219.

8. Dessauer, H. C., Fox, W., *Science,* 1956, v124, 225.

9. Frieden, E., Herner, A. E., Fish, L., Lewis, E. J. C., *ibid.,* 1957, v126, 559.

10. Zweig, G., Crenshaw, J. W., *ibid.,* 1957, v126, 1065.

11. Cohen, E., Stickler, G. B., *ibid.,* 1958, v127, 1392.

12. Dessauer, H. C., Fox, W., Proc. Soc. Exp. Biol. and Med., 1958, v98, 101.

13. Engle, R. L., Jr., Woods, K. R., Paulsen, E. C., Pert, J. H., *ibid.,* 1958, v98, 905.

14. Baril, E. F., Palmer, J. L., Bartel, A. H., *Science,* 1961, v133, 278.

15. Rall, D. P., Schwab, P., Zubrod, C. G., *ibid.,* 1961, v133, 279.

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Endogenous Cathepsin Activation of Trypsinogen in Extracts of Dog Pancreas.* (26539)

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The pancreas and pancreatic juice normally contain negligible amounts of active trypsin (1). Trypsin, in the form of its zymogen trypsinogen, is secreted into the intestine where the activation of trypsinogen to trypsin is catalyzed by the enzyme enterokinase. Once formed, trypsin may also catalyze the formation of trypsin from trypsinogen. The mechanism by which the extracellular enzymes enterokinase and trypsin activate trypsinogen has been established as the hydrolytic cleavage of a hexapeptide from the amino terminal portion of the trypsinogen molecule(2,3). The only intracellular mammalian enzyme that has been demonstrated to activate trypsinogen is the partially purified enzyme cathepsin B(4,5). Our previous *in vitro* studies have shown that beef spleen cathepsin B purified 200 fold, catalyzes the activation of crystalline trypsinogen to trypsin (or a trypsin-like enzyme) as determined by the action of the active product against synthetic peptide substrates and its inhibition by trypsin-soy bean inhibitor(4,5). These studies demonstrated that optimum pH for this activation occurred at pH 3.6, a pH at which no trypsin activation of trypsinogen occurs. It was further demonstrated that addition of .001 M iodoacetic acid (a known inhibitor of cathepsin B) or omission of cysteine (a known activator of cathepsin B) inhibited the reaction.

In view of the intracellular location of cathepsin B(6), it was of interest to deter-

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mine whether there are present in the pancreas cathepsin B or enzymes similar in nature which can activate the endogenous trypsinogen. In the present study, extracts and concentrated extracts of dog pancreas were prepared which were demonstrated to contain materials which activate the endogenous trypsinogen in these extracts to trypsin (or a trypsin-like enzyme) under conditions similar to those in which cathepsin B activates trypsinogen. Activation of trypsinogen was enhanced by the presence of cysteine and inhibited by iodoacetic acid. These findings may be of interest in relation to diseases such as pancreatitis where the possibility of abnormal trypsin formation in the pancreas may occur, although this point has not been definitely established(7).

Methods. Extracts of dog pancreas were prepared as follows: All steps were carried out at 2°. 20-40 g of dog pancreas were homogenized in a Waring blender for 2 minutes with 2 to 4 volumes of 2% NaCl and .001 M versene in 0.015 N hydrochloric acid. The suspension was brought to pH 2.5 with 1 N HCl with stirring. 0.5 ml of toluene was added and the suspension placed in the refrigerator for 20 hours. The suspension was centrifuged at 16,000 rpm for 30 minutes in the cold and the supernatant fluid obtained was used in these experiments and is termed the "extract". In some experiments the supernatant fluid was saturated with ammonium sulfate (69 g ammonium sulfate/100 ml extract at 2°), the resulting suspension was centrifuged for 15′, and the precipitate suspended in a small amount of .001 M citrate solution at pH 3.6. Ammonium sulfate was removed by dialysis against citrate buffer at pH 3.6. The dialyzed solution is termed the "concentrated extract". Cathepsin B was prepared as previously described(8). Trypsinogen was the 1 × crystalline product of Worthington Biochemical Corp. purified as previously described(5). Trypsin soy bean inhibitor and pancreatic trypsin inhibitor complex were products of The Worthington Biochemical Corp. Trypsin assays were carried out by the spectrophotometric technic against benzoyl-l-arginine ethyl ester also previously described (5).

TABLE I. Increase in Trypsin Units* from Zero Time.†

Incubation mixture‡	△ 20 min.	△ 40 min.	△ 120 min.
Extract alone	—	—	36
Extract + .001 M iodoacetic (pH 3.6)	—	—	0
Extract + trypsinogen§	—	—	53
Concentrated extract	53	103	
Idem + iodoacetic	18	34	
" − cysteine	29	53	
" + soy bean inhibitor‖	10	26	

* A trypsin unit is defined as that amount of trypsin that produces an increase in .001 absorbance units/min. when assayed against benzoyl-l-arginine ethyl ester at 253 mμ under the conditions set forth previously(5). Values expressed as trypsin units/ml incubation mixture. A trypsin unit is approximately equal to .001 mg crystalline trypsin when assayed against benzoyl-l-arginine ethyl ester(5).

† All extracts had zero time activity, attributed to the fact that extraction procedures were carried out at pH 3.6. Zero time activity was subtracted from final activity to give values in the table.

‡ Incubation mixture consisted of 0.6 ml "extract" or 0.25 ml "concentrated extract" in .04 M acetate, .04 M cysteine, .08 M $CaCl_2$ to a total of 1 ml pH 3.6 at 25°.

§ 4.0 mg of crystalline trypsinogen in 1.0 ml of incubation mixture.

‖ 3× the weight (in mg) of trypsin present in incubation mixture at 40′.

Results and discussion. As can be seen from a typical experiment in Table I, when pancreatic extracts are allowed to incubate at pH 3.6 for 2 hours there is an increase in endogenous trypsin activity with time. In the presence of iodoacetic acid no increase in activity was observed. The inhibition of trypsin formation by iodoacetic acid demonstrates that activation of endogenous trypsinogen is mediated by materials sensitive to iodoacetic acid and not by any trypsin present in these extracts since the latter enzyme is not affected by iodoacetic acid. When crystalline trypsinogen is added to these extracts, a further increase in trypsin activity occurs with time over that of the extract alone. This is further proof of the presence of trypsinogen activating material in these extracts. Control experiments with crystalline trypsinogen alone under these conditions demonstrated no increase in trypsin activity. Incubation mixtures of crystalline trypsin and trypsinogen under these conditions also produced no increase in trypsin over that of the trypsin added. Control experiments with pancreatic trypsin inhibitor complex(9) demonstrated that this complex does not dissociate under the conditions of the experiments.

To reduce incubation times, concentrated extracts were prepared as described above. Measurable quantities of trypsin were formed by these extracts in 40 minutes. When iodoacetic acid is added to these extracts or when cysteine is omitted there is again a reduction in trypsin formation. Addition of crystalline trypsin soy bean inhibitor to these extracts reduced the trypsin activity formed, demonstrating that trypsin activity is actually being measured in these experiments. Addition of cathepsin B to extracts similar to those shown produced an increase in trypsin activity over that of the extract alone. This is further proof of the presence of trypsinogen in these extracts and that these extracts contain no materials particularly inhibitory to the action of enzymes like cathepsin B towards trypsinogen.

On the basis of the cysteine and iodoacetic acid sensitivity and formation of trypsin at pH 3.6, it is reasonable to assume that activation of trypsinogen in these extracts is mediated at least in part by cathepsin-like enzymes, the same or similar to cathepsin B. The complex enzymatic activity of these extracts, however, makes necessary further investigation with large quantities of these extracts to define precisely the cathepsin activity. Nevertheless, it is of interest that materials other than trypsin are present in the pancreas which under proper conditions activate endogenous trypsinogen to trypsin. Such reactions may be important in certain diseases of the pancreas in which the structural integrity of the pancreas is destroyed so that the zymogen granules containing trypsinogen, normally protected from the action of intracellular proteases, may be brought in contact with these proteases causing activation. This would lead to proteolytic destruction of the pancreas and release of the proteases into the blood stream as is found in cases of pancreatitis(10).

Summary. Evidence is presented that endogenous trypsinogen in extracts of dog pancreas is activated under the same conditions that cathepsin B activates trypsinogen *in vitro*. The activation occurs at pH 3.6 and is enhanced by cysteine and is inhibited by iodoacetic acid. The tentative conclusion reached is that cathepsin B-like enzymes in pancreas are catalyzing the reaction. The possible clinical implications of such reactions is discussed.

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1. Keller, P. J., Cohen, E., Neurath, H., *J. Biol. Chem.*, 1958, v233, 344.

2. Davie, E. W., Neurath, H., *ibid.*, 1955, v212, 515.

3. Yamashina, I., *Biochim. et Biophysica Acta*, 1956, v20, 433.

4. Greenbaum, L. M., Hirshkowitz, A., *Fed. Proc.*, 1958, v17, 234.

5. Greenbaum, L. M., Hirshkowitz, A., Shoichet, I., *J. Biol. Chem.*, 1959, v234, 2885.

6. Fruton, J. S., In Boyer, P. D., Lardy, H., Myrback, K., (Editors) *The Enzymes* Vol. 4, Academic Press, New York, 1960, p233.

7. Grossman, M. I., *J. Am. Med. Assn.*, 1959, v169, 1567.

8. Greenbaum, L. M., Fruton, J. S., *J. Biol. Chem.*, 1957, v226, 173.

9. Northrop, J. H., Kunitz, M., Herriott, R. M., *Crystalline Enzymes*, 2nd Edition, Columbia Univ. Press, New York, 1948.

10. Nardi, G. L., Lees, C. W., *New Eng. J. Med.*, 1958, v258, 797.

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Effect of D-Amphetamine on Rat Brain Noradrenaline and Serotonin. (26540)

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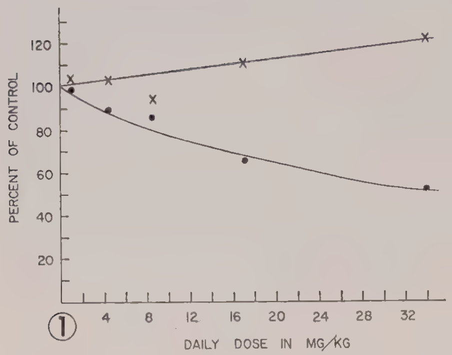
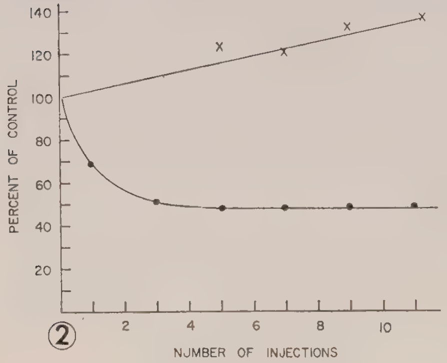
Recent interest in the role of noradrenaline and serotonin as possible neurohumors has stimulated investigation of the influence of various treatments on the biochemistry of these metabolites. Experimental conditions having differential effects on tissue levels of noradrenaline and serotonin may be of particular value in understanding the possible functions of these compounds in brain metabolism. The sedative reserpine, for example, depresses brain levels of both amines; however, further observations suggest that the effects of Rauwolfia alkaloids may be related to the changes in serotonin rather than noradrenaline. In rats and rabbits treated with a reserpine analogue, or cold stressed and treated with reserpine, noradrenaline levels were markedly depressed with a much smaller change in serotonin, and sedation was not produced(1). Iproniazid and other monoamine oxidase inhibitors elevated both noradrenaline and serotonin in rat and rabbit brain and caused central excitation, whereas in cats and dogs only serotonin was markedly elevated and pronounced central excitation was not produced (2). Vogt reported that in several species, drugs causing hypothalamic stimulation lowered hypothalamic noradrenaline levels(3). During a study of the effects of a number of drugs on rat brain metabolism it was noted that repeated injections of amphetamine or ephedrine lowered noradrenaline levels. The results of a further study with D-amphetamine are given in this report.

Materials and methods. Drugs were given twice daily at 8 a.m. and 4 p.m. by subcutaneous injection to groups of 5-10 male Holtzmann rats weighing 170-200 g. The animals were killed by decapitation, the whole brain removed and immediately frozen on dry ice. Except where otherwise noted the rats were killed 1 hour after last injection. The tissue was extracted as described by Shore and Olin(4), and noradrenaline determined fluorometrically by the method of Von Euler and Floding(5) using an Aminco-Bowman spectrophotofluorometer, with activation at 390 mμ and fluorescence measurement at 490 mμ. Serotonin was also determined in the same acid extract by the fluorometric method of Bogdanski *et al.*(6). For noradrenaline, the average value obtained with 8 separate groups of control animals was .32(.19-.50) γ per g wet weight. For serotonin, the average value obtained was .58(.45-.72) γ per g wet weight. The variation in values obtained with samples of the same homogenate extracted and assayed together was ± 8%. Where several treatments were compared the tissues were extracted and assayed together. Monoamine oxidase was determined by Warburg assay using tyramine as substrate.

Results. Progressive doubling of the daily doses of D-amphetamine caused progressively greater decreases in brain noradrenaline levels and a slight increase in serotonin levels (Fig. 1). Fig. 2 shows the effects of from one to 11 injections of 17 mg/kg of D-amphetamine. Noradrenaline levels reached a minimum after 5 injections, serotonin levels continued to rise slightly with repeated injections. Lowering of brain noradrenaline and elevation of serotonin persisted for at least 120 hours after last injection of D-amphetamine (Fig. 3). Table I shows the effect of combined treatment with iproniazid and amphetamine. There was no detectable monoamine oxidase activity in the brains of animals treated with iproniazid, alone or with amphetamine. Iproniazid alone gave the expected increase in both noradrenaline and serotonin. D-Amphetamine antagonized the elevation of noradrenaline by iproniazid, and moderately enhanced the elevation of serotonin.

Discussion. It does not seem probable that the decrease in noradrenaline following D-amphetamine administration is due to any change in an exchange across the blood brain





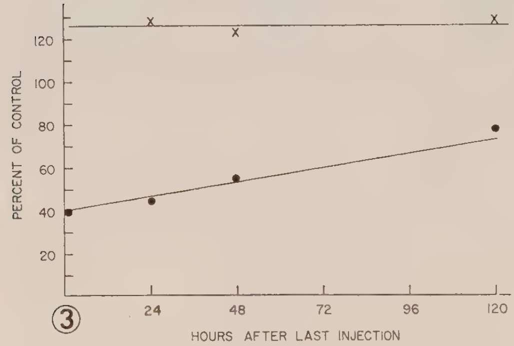


FIG. 1. Brain noradrenaline ●—●, and serotonin ×—×, in rats given D-amphetamine sulfate, 2 subcut. injections a day for 2 days and 1 injection on the third day.

FIG. 2. Brain noradrenaline ●—●, and serotonin ×—×, in rats given twice daily subcut. injections of 17 mg/kg of D-amphetamine sulfate.

FIG. 3. Brain noradrenaline ●—●, and serotonin ×—×, in rats given D-amphetamine sulfate, 2 subcut. injections a day for 2 days, 1 injection on the third day, then killed at intervals thereafter.

barrier. Although there is no direct evidence on losses of brain noradrenaline to the blood stream, injected noradrenaline is not taken up from the blood at an appreciable rate(7). It has been suggested that monoamine oxidase is a major pathway for degradation of noradrenaline in brain(8). The level of noradrenaline in animals treated with iproniazid plus amphetamine is lower than the level in animals given iproniazid alone (Table I). Since monoamine oxidase is completely inhibited in these animals it does not appear that the effect of amphetamine in lowering noradrenaline levels is due to any effect on monoamine oxidase.

TABLE I. Brain Noradrenaline and Serotonin Levels in Rats Given Twice Daily Injections of Iproniazid Alone or with D-amphetamine for 3 Days.

Drug	Dose in mg/kg/day	Brain level as % of control: Noradrenaline	Brain level as % of control: Serotonin
Iproniazid	20	179	230
D-amphetamine sulfate + Iproniazid	34 20	100	280
D-amphetamine sulfate + Iproniazid	17 20	135	290

A further possibility is that the decrease in brain noradrenaline following treatment with amphetamine could be due to a decrease in synthesis. It has been suggested that amphetamine mimics the action of noradrenaline centrally(9). If this is true one might expect prolonged treatment with amphetamine to activate a feed back mechanism depressing the synthesis of noradrenaline. In any case, the differential effect of D-amphetamine on noradrenaline and serotonin may serve as another illustration of the existence of independent metabolic pathways for the 2 postulated neurohormones.

Summary. The treatment of rats with D-amphetamine sulfate lowered brain noradrenaline and slightly elevated brain serotonin. These effects lasted for several days after discontinuing treatment. D-amphetamine sulfate antagonized the elevation of brain noradrenaline levels by iproniazid.

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1. Brodie, B. B., Finger, K. F., Orlans, F. B., Quinn, G. P., Sulser, F., *J. Pharm. Exp. Therap.*, 1960, v129, 250.

2. Spector, S., Shore, P. A., Brodie, B. B., *ibid.*, 1960, v128, 15.

3. Vogt, M., *J. Physiol.*, 1954, v123, 451.
4. Shore, P. A., Olen, J. S., *J. Pharm. Exp. Therap.*, 1958, v122, 295.
5. Von Euler, U. S., Floding, I., *Acta Physiol. Scand.*, 1955, Suppl. 33, v118, 45.
6. Bogdanski, D. F., Pletscher, A., Brodie, B. B., Udenfriend, S., *J. Pharm. Exp. Therap.*, 1956, v117, 82.
7. Schayer, R. W., *J. Biol. Chem.*, 1951, v189, 301.
8. Grout, J. R., Creveling, C. R., Calon, D., *Fed. Proc.*, 1960, v19, 297.
9. Brodie, B. B., Shore, P. A., *Ann. N. Y. Acad. Sci.*, 1957, v66, 631.

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Biological Assay of Proteolytic Enzymes Capable of Debriding Third Degree Burn Eschars. (26541)

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In attempting to identify the proteolytic enzyme or enzymes in *Clostridium histolyticum* culture filtrates which were responsible for the ability of these preparations to debride third degree burn eschars, it was found that adequate methods of bioassay were lacking. Although in recent years a variety of enzymes have been proposed as agents for removal of burn slough, methods for comparing the biological potency of these enzymes have not been available. Mention has been made of the use of laboratory animals such as the guinea pig(1,2), dog(3,4) or rabbit (5,6) but no attempt has been made to develop a quantitative procedure.

The present paper describes a method for biological assay of proteolytic enzymes capable of debriding third degree burn eschars. A suitable bandaging technic for maintaining the enzyme solution in contact with the burned surface was developed and a scale for assessing the effectiveness of debridement was chosen. In addition, 2 examples of the use of this assay are given; 1) evaluation of debriding activities of various enzyme preparations, and 2) determination of the effect on debriding activity of pre-treatment of the burn eschar with pHisoHex and with petrolatum.

Materials and methods. Four proteolytic enzymes were used. Two of the enzyme preparations, designated as 0-22% enzymes and 30-35% enzymes, were obtained from *C. histolyticum*, H-4, filtrates by a modification of the procedure developed by deBellis *et al.* (7). The mold proteinase from culture X-108 (Lot No. 7-1162D-12) was kindly supplied by Frank B. Ablondi, Biochemical Research Section, Research Division, American Cyanamid Co. Purified crystalline Trypsin (approximately 50% $MgSO_4$, Lot TP11) was purchased from Worthington Biochemical Laboratory. All enzyme preparations were dissolved in 0.1 M tris (hydroxymethyl) aminomethane buffer, pH 7.5, containing 0.01 M $CaCl_2$ and 0.5% proteose peptone (Difco), except the mold enzyme which was dissolved in 5% proteose peptone, pH 7.5. In addition, 1000 units of potassium penicillin G and 500 μg of streptomycin were added for each ml of test solution to inhibit bacterial contamination.

Hartley strain guinea pigs (350-800 g) were anesthetized with sodium pentobarbital and the entire dorsal area was clipped as closely as possible. Four full thickness third degree burns with a diameter of approxi-

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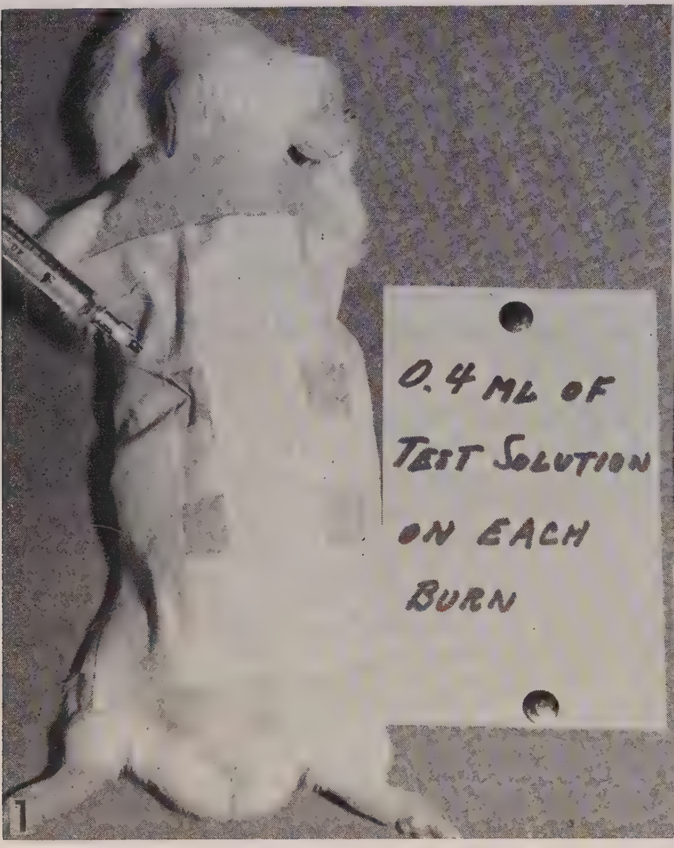

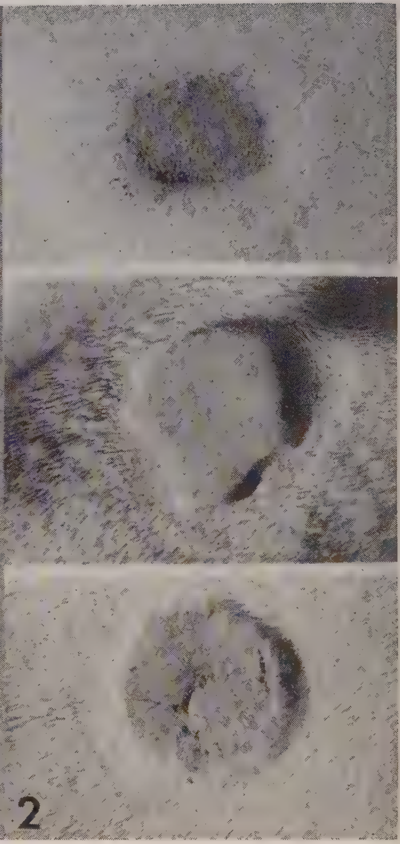

FIG. 1. Typical method of bandaging of guinea pigs for assay of ability of proteolytic enzymes to debride third degree burn eschars.

FIG. 2. Surface of burn eschar after application of test solutions. Top: Buffer, 0% debridement. Middle: 16 azocasein units 0-22% enzymes, 37.5% debridement. Bottom: 400 azocasein units 0-22% enzymes, 100% debridement.

mately 1 cm were produced on the shaved area with a Stanley No. 435 80 watt soldering iron, the tip of which had been filed down to give an oval surface approximately 10 × 6 mm. The hot iron (360-400°C) was applied for 15 seconds with sufficient pressure to insure good contact without making a depression in the skin of the guinea pig. Charred tissue was removed from the tip of the iron between each burn. Unventilated 1½″ × 1½″ plastic patches, kindly supplied by Johnson & Johnson Co., were carefully laid over each burn so that the small gauze pad would be directly over and in contact with the burn. The edges of the patches were sealed and reinforced with strips of adhesive tape. With a hypodermic syringe and needle, 0.4 ml of test solution was inoculated into each of the 4 plastic patches on a guinea pig, as shown in Fig. 1. The opening made by the needle was sealed with a small piece of plastic tape or with a drop of Duco cement.

After 18 hours the guinea pigs were reanesthetized and the bandages removed. All burns were examined to check whether the sites were moist and the assay results valid; occasionally a corner of the bandage became loosened permitting the site to dry, or the patch became elevated over the burn giving poor contact and consequently poor debridement. The digested tissue was removed by wiping the treated area with cotton balls held in forceps, rubbing with moderate pressure in all directions. The enzymes do not remove the burn tissue, but rather soften the eschar so that it can be removed by wiping. If the surface of the burn becomes dry after digestion by the enzymes, the slough hardens and

TABLE I. Influence of Weight of Guinea Pigs and Concentration of Enzymes on Debridement.

Conc. of 0-22% proteinases	Wt of guinea pigs in g 793 ± 26	 589 ± 46	 388 ± 33
Azocasein units		Avg % debridement	
160	42	55	69
80	41	45	63
40	28	31	56
Buffer	0	0	0

Weight investigated by joining of 3 Latin squares, each square representing 4 guinea pigs (columns) of same weight with 4 sites (rows) per guinea pig(8). Three dilutions of enzyme were tested together with a buffer control. Two males and 2 females were used to form each Latin square in lower weight groups. Experiment was replicated on the following day.

can no longer be removed. Therefore, it is necessary that the burn eschar be kept moist with the enzyme solution. When high concentrations of enzyme were applied to the burn sites in an attempt to obtain 100% debridement, leakage to adjacent control sites occurred; this could be eliminated by sealing the edges of the plastic patches with Duco cement. Comparison of values obtained which were below 75% suggested that the results with either method of sealing the bandage were similar, indicating that leakage had no appreciable effect when lower enzyme concentrations were used. It was also found that debridement greater than 75% was not necessarily proportional to enzyme concentration. Therefore, the rapid technic with adhesive tape alone was utilized in these studies. Biological activity was recorded on a scale ranging from 0 to 8 in the following manner: 0 (0%), no effect after rubbing 10-20 times; 1 (12.5%), slight break in eschar; 2 (25%), surface removal of eschar; 3 (37.5%), partial removal of eschar to corium; 4 (50%), complete removal of eschar to corium; 5 (62.5%), partial removal of eschar to panniculus carnosus; 6 (75%), complete removal of eschar to panniculus carnosus except at edges of burn; 7 (87.5%), partial removal of denatured tissue at edges of burn; 8 (100%), complete removal of all devitalized tissue (Fig. 2).

Results. It was found (Table I) that differences in weight of the test guinea pigs greatly influenced the amount of enzyme required to accomplish debridement; 40 units on guinea pigs weighing 388 g gave more complete debridement than 160 units on guinea pigs weighing 793 g. Presumably older animals with thicker skins furnished an increased amount of substrate for the enzyme to digest. Two-fold enzyme concentrations did not always give significantly different results but 4-fold enzyme concentrations could readily be distinguished.

Degree of debridement was not influenced by sex of the animal, but was significantly affected by the location of skin area tested, greater debridement occurring at the 2 top sites than at the 2 bottom sites (Table II). Whether these differences are due to variation in skin thickness at these locations or to a greater leak rate occurring from the bandage at the lower sites is not known. Nonetheless, for accurate determination of enzymatic activities the effect of sites must be controlled in the experimental design.

On the basis of the results described above, a standard method was adopted for comparing the ability of various proteolytic enzyme preparations to digest third degree burn eschars. Experimental burns were produced, as previously described, on the backs of Hartley strain guinea pigs of similar weights. Appropriate dilutions of the enzyme preparations under investigation were applied under the plastic patches to the burn sites. Enough animals were used to permit testing of each preparation on each of the 4 available sites, a different location on each animal. The readings of degree of debridement at each of the treated sites were averaged.

With this method, a comparison was made

TABLE II. Influence of Sex and Sites on Debridement.

Variable		Avg % debridement
Sex	Female	41
	Male	39
Sites	Left top	39
	" bottom	33
	Right "	30
	" top	41

Data from experiment in Table I. Avg of responses furnished by 3 concentrations of enzyme.

TABLE III. Comparison of Ability of 4 Proteinases to Debride Burns.

Proteolytic enzymes	Concentration applied			
	Units*	Azocasein units*	Protein (mg)†	Avg % debridement
Mold	1,600	72	.007	62
0-22%	80	80	.027	61
Trypsin	1,600	760	.200	56
30-35%	30	6	.047	54

* Units applied are those of manufacturers. Substrate for mold enzyme was azocoll; for trypsin, N-benzoyl-L-arginine ethylester; for 0-22%, azocasein and for 30-35%, gelatin. Azocasein units furnish comparison of these proteinases on this substrate.

† Calculated from nitrogen determination.

Results obtained by joining of 2 Latin squares. Experiment was replicated on another day.

of the ability of 4 proteolytic enzyme preparations to debride third degree burns (Table III). On the basis of their protein content, the highly purified mold proteinase was a more effective debriding agent than the 0-22% and 30-35% proteinases from *C. histolyticum*. Trypsin, on the other hand, required much higher concentrations than the other enzymes to digest this substrate and it is apparent that the ability of these proteinases to attack azocasein does not parallel their ability to debride a third degree burn eschar.

To determine the effect of pre-treatment of the burn eschar on the debriding activity of *C. histolyticum* proteinases, 2 substances which are commonly used to treat burns in human patients were studied; pHisoHex, which is used to cleanse the burn, and petrolatum, which is used to protect the eschar from air. pHisoHex was a potent inhibitor of the debriding activity of the 0-22% proteinase (Table IV). Washing the burn to remove pHisoHex before application of the enzyme effectively removed the inhibitor. Petrolatum under these conditions did not inhibit enzymatic digestion.

Discussion. This study has demonstrated that a semi-quantitative *in vivo* assay for measuring debriding activity of various proteinase preparations is practical. Antibiotics or other bacteriostatic or bactericidal agents must be added to the enzyme solution under test to prevent contaminating bacteria from causing false-positive results. The need for this combination of enzymes and antibiotics in the human patient has been adequately discussed by Greuer and Hess(9). Removal of the bulk of the dead tissue by enzymatic debridement carried out in the presence of antibiotics may prove to be of value in reducing the late deaths in burns due to bacterial infection. The proteolytic enzymes used in these studies, when applied topically and in concentrations suitable for removal of the eschars, did not digest normal skin and apparently limited their activity to digestion of denatured tissue. No gross inflammatory changes beyond the margin of such burns have been observed.

The use of statistical designs such as Latin squares, Youden squares, Lattice squares or other designs where the units per block are equal to 4 is especially valuable in this bioassay in controlling biological and spatial variation. A difficulty in application of statistics to biological results is the mathematical need for additivity of the data. Thus the natural desire of the biologist to use a buffer control as one of the treatments in the experimental design results in overestimating the significance of the statistical analyses. This may also occur when a given treatment (Table IV) causes complete inhibition of enzymatic activity resulting in zero variance. Nevertheless treatment means obtained by

TABLE IV. Effect of pHisoHex and Petrolatum on Debridement.

Treatment of burn	0-22% enzymes, applied units	Avg % debridement
None	200	72
"	80	63
pHisoHex	80	0
" *	80	63
Petrolatum	80	72
" *	80	72
None	Buffer	0

* After 24 hr pHisoHex and petrolatum were removed by washing with lukewarm water and 95% ethanol respectively.

Youden square statistical design. Seven treatments occurred once in each column (site) and each pair of treatments appeared together an equal number of times in the same block (guinea pig). Animals burned as usual and pHisoHex and petrolatum applied to appropriate site.

use of the statistical experimental design are unquestionably more accurate than if no design had been utilized.

In the bioassay procedure described a standard method of burning (contact) and a standard laboratory animal (guinea pig) have been chosen. Additional studies will be required to indicate whether the same burn on other animals requires the same enzymes or, conversely, whether different types of burns on the same laboratory animal are as readily debrided.

Summary. A semi-quantitative bioassay has been described for determining the ability of proteolytic enzymes to debride third degree burn eschars on guinea pigs. With the aid of statistical designs, body weight and site of application were established as the 2 most important experimental variables. Trypsin, a highly purified mold enzyme, and 2 proteinase preparations from *C. histolyticum* have been compared for their ability to debride contact burns. pHisoHex was found to inhibit the 0-22% proteinases.

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1. Altemeier, W. A., Coith, R., Culbertson, W., Tytell, A., *Ann. Surgery*, 1951, v134, 581.

2. Connell, J. F., Rousselot, L. M., *S. Forum*, 1953, v4, 422.

3. May, S. C., Ziffren, S. E., Kallio, R. E., Larson, A. D., *ibid.*, 1953, (1952), 205.

4. Stucke, K., *Chirurg.*, 1954, v25, 289.

5. Howes, E. L., *N. Y. State J. Med.*, 1944, v44, 2006.

6. Howes, E. L., MacLennan, J. D., Mandl, I., DeBellis, R., *Bull. N. Y. Acad. Med.*, 1955, v31, 413.

7. DeBellis, R., Mandl, I., MacLennan, J. D., Howes, E. L., *Nature*, 1954, v174, 1191.

8. Cochran, W. G., Cox, G. M., *Experimental Designs*, John Wiley & Sons, Inc., New York, 1950.

9. Greuer, W., Hess, E., *Arzeneimihel-Forsch.*, 1954, v4, 432.

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Deposition of Cholesterol and Cholestanol in Experimental Rabbit Atherosclerosis.* (26542)

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When cholesterol-C^{14} (prepared biosynthetically from acetate-1-C^{14}) and cholesterol-H^3 (prepared by the Wilzbach method) were fed to rabbits(1), a striking discrepancy was observed in the counts of cholesterol samples isolated from tissues like aortae, livers or small intestines. Those isolated from cholesterol-H^3 fed animals showed a much lower radioactivity transfer than those from cholesterol-C^{14} fed rabbits. Since the C^{14} and H^3 cholesterols both appeared to be radiochemically homogeneous (having been subjected to bromination and various chromatographic procedures respectively) it was suggested that the difference in behavior between these 2 labelled cholesterols might have been the result of a peripheral oxidation of cholesterol during its stay in the body, eliminating H^3 atoms from the molecule. Shortly after these experiments were finished, a report of findings by Nystrom and Sunko(2) about the addition of tritium to the double bond of cholesterol when Wilzbach's method of tritiation(3) was used, raised the question whether cholestanol-H^3 was a contaminant in our previous cholesterol-H^3 sample, despite the purification technics we had used. Since cholestanol-H^3 might be metabolized differently from cholesterol-H^3 and might be responsible for the differences of radioactivity observed in earlier experiments, it was decided to repeat the earlier feeding experiments using cholesterol-H^3 purified in such a way as to eliminate all cholestanol-H^3 formed during tritiation. Special work showed that it is possible to eliminate completely cholestanol-H^3 by repeated bro-

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mination and debromination of cholesterol-H^3.

To test the assumption that cholestanol-H^3 present in the cholesterol-H^3 fed was the cause of the discrepancy observed in the earlier experiments, pure cholestanol-H^3 was prepared by hydrogenation of cholesterol-H^3 with non-radioactive hydrogen and fed to rabbits in a separate experiment.

Methods. Cholesterol-C^{14} was obtained from rats after injection of sodium acetate-1-C^{14} and cholesterol-H^3 was prepared by treatment of highly purified cholesterol or its acetate with tritium according to Wilzbach, stabilized by boiling with alkali in methanol and then after appropriate dilution with pure cholesterol purified completely by bromination and debromination and recrystallization from methanol-ether(4). These procedures were repeated until the isolated pure cholesterol-H^3 fractions showed the same constant count as the corresponding fractions from the bromination mother liquors. Four such brominations were required. These procedures will be described in a later report.

Experiment 1. Five male and 5 female New Zealand white rabbits weighing 3-4 lb were fed for 12 weeks 900 mg per day and per animal of commercial cholesterol (Wilson), mixed with their normal food. Two of the animals then received, for 6 days instead of the commercial material, 900 mg cholesterol-C^{14}. The other 8 were fed 900 mg pure cholesterol-H^3 for 6 days in addition to their normal rations. On the day after the last feeding of radioactive material all animals were killed and blood taken for a serum cholesterol determination. The aortae, livers and small intestines were combined for each group and cholesterol isolated as reported before(5). All animals showed gross atherosclerotic lesions and their serum cholesterol varied from 300 to 1300 mg%.

Experiment 2. Four male and 3 female New Zealand white rabbits weighing 3-4 lb were fed each for 6 weeks per day 900 mg commercial cholesterol. During the seventh week they received daily a mixture of 810 mg nonradioactive pure cholesterol and 90 mg pure cholestanol-H^3 which according to a Liebermann - Burchard determination contained 0.35% cholesterol-H^3. In these 900 mg of mixture cholestanol-H^3 made nearly 10% of the total. The count of this cholestanol-H^3 was 3.8×10^4 per min per mg while the count of the cholesterol-H^3 was only 1.44×10^2 per min per mg. The rabbits were killed after 6 daily feedings. They showed atherosclerotic lesions and their blood serum cholesterols were from 276 to 600 mg%. Aortae, livers and gastrointestinal tracts were treated as in the other series.

Results are presented in Tables I and II. Contrary to the previous results, in Experiment I (Table I) the dilution in the tissues was essentially the same for C^{14} or H^3 cholesterol, showing that both types of cholesterol, as should be expected, were deposited in the same manner. Appropriate calculation shows that about 0.06 and 0.02 mg respectively of cholesterol were laid down in the aorta per day per animal and per gram of aorta tissue, a value of the same magnitude as found in the earlier experiments (0.03 mg). It is evident therefore that the disappearance of radioactivity reported earlier(1) was not caused by a peripheral oxidation, but by the presence of a higher than cholesterol-H^3 counting companion which was deposited in the tissues only in very small amounts if at all. That the cholesterol-H^3 used in the present investigation was free from cholestanol-H^3 suggests strongly that the higher counting companion in earlier experiments was cholestanol-H^3. Experiment 2 confirms this explanation (Table II). The mixture fed here contained the bulk of radioactivity in the form of cholestanol-H^3, but only a very small part of this radioactivity appears in the cholesterol isolated from the tissues when the dilution figures are compared with the dilution in Table I. It is evident that what is laid down is mainly cholesterol but as observed earlier (6) cholestanol is definitely also deposited in the tissues. It is also clear from the dilution figures that deposition of cholesterol and cholestanol is quite different for different tissues. The lesser dilution of the fed material in the small intestine shows that considerably more of both substances is retained there than in

TABLE I. Feeding of Cholesterol-C^{14} and Cholesterol-H^3 to Rabbits.

	2 rabbits fed cholesterol-C^{14} with 3734 counts/min./mg				8 rabbits fed cholesterol-H^3 with 16408 counts/min./mg			
		Cholesterol-C^{14} isolated				Cholesterol-H^3 isolated		
	mg cholesterol/g wet tissue	Count per min./mg	Dilution*	mg cholesterol-C^{14} laid down per day/rabbit/g wet tissue	mg cholesterol/g wet tissue	Count per min./mg	Dilution*	mg cholesterol-H^3 laid down per day/rabbit/g wet tissue
Aorta	3.8	663	5.6	.056	3.6	3586	4.6	.018
Liver	3.7	1196	3.1	.01	3.3	5956	2.8	.033
Small intestine	1.5	1544	2.4	.049	2.8	7485	2.2	.031

* Dilution gives the relation between counts of cholesterol fed, divided by counts of cholesterol isolated from tissues, both counted in a scintillation counter. Cholesterol-C^{14} samples were also counted with an end window counter on which the starting material had 244 counts/min./mg. Figures obtained for the dilution in this case were 4.9, 2.8 and 2.2, identical with the figures in the cholesterol-H^3 experiment.

TABLE II. Feeding a Mixture of Cholesterol-H^3 and Cholestanol-H^3.

7 rabbits fed: Cholesterol-H^3 with 1.44×10^2 counts/min./mg
Cholestanol-H^3 with 3.8×10^4 counts/min./mg

		Sterol-H^3 isolated*				
	mg sterol isolated per g wet tissue	Counts per min./mg	Dilution total of sterol-H^3	Dilution calculated† for Cholesterol-H^3	Cholestanol-H^3	mg sterol-H^3 laid down per day/rabbit/g wet tissue
Aorta	2.1	980	388	5	400	.014
Liver	5.4	1008	377	3	396	.039
Small intestine	2.2	3600	105	2	11	.046

* Total sterol isolated from digitonide precipitation of extracts from tissues.
† Figures for dilution of cholesterol-H^3 were taken from Table I and used to calculate dilution for cholestanol-H^3.

the aortae and livers. This phenomenon is evidently connected with the passage of these substances through the intestinal wall.

Summary. Contrary to the original findings it is established that cholesterol-C^{14} and cholesterol-H^3 are deposited in tissues of rabbits in exactly the same way. The reason for the discrepancy in deposition of the two substances in earlier experiments was found in the presence of chemically negligible traces of cholestanol-H^3 which had then not been eliminated from cholesterol-H^3 fed. Deposition is different for different tissues and that of cholestanol is only a very small fraction of that for cholesterol.

The help of Dean F. Stevens and Eva Morin is gratefully acknowledged.

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1. Schwenk, E., Stevens, D. F., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 614.
2. *Atomlight,* issued by New England Nuclear Corp., Jan. 1959, p1.
3. Wilzbach, J., *J. Am. Chem., Soc.,* 1957, v79, 1013.
4. Fieser, L. F., *ibid.,* 1953, v75, 5421.
5. Schwenk, E., Alexander, G. J., Fish, C. A., *Arch. Biochem. & Biophys.,* 1955, v58, 37.
6. Cook, R. P., *Cholesterol,* Academic Press, Inc., N. Y., 1958, p245.

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Some Metabolic Properties of the Rabbit Oviduct.* (26543)

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The human endosalpinx displays some unusual metabolic characteristics *in vitro*(1). It resembles certain malignant tissues in that there is a substantial accumulation of lactic acid when it is incubated aerobically with glucose. In the present experiment some *in vitro* metabolic properties of the rabbit oviduct are explored. The rabbit offers the advantage that hormonal conditions can be controlled, and an accurate correlation between *in vitro* metabolic activity and the endocrinological status of the animal is possible.

Materials and methods. New Zealand White rabbits weighing 3-5 kg were used throughout. Animals were housed in separate cages and were given a diet of Purina chow and water *ad libitum*. Oviducts were removed under sodium pentobarbital anesthesia and placed immediately in cold Krebs-Ringer phosphate solution (K.R.p.). Specimens were freed of surrounding tissues by careful dissection, supported with pith and sliced transversely into thin sections. These were studied at 37°C in the Warburg apparatus in K.R.p. alone and with added substrates. After 2 hours' incubation, hexose disappearance and lactic acid accumulation were determined. Nelson's modification of the Somogyi method(2) was used for sugar determinations and the method of Barker and Summerson(3) for lactate determinations. The tissue in each flask was dried overnight at 106°C and weighed. Results were expressed as μg/mg dry weight. Intact non-pregnant animals, castrates and mated animals 16-72 hours following coitus were selected for study. In the intact non-pregnant group, no attempt was made to distinguish between anestrous and estrous rabbits. Those whose ovaries contained obvious corpora lutea were excluded. The castrates were ovariectomized 23-49 days prior to study. In mated animals, coitus was confirmed by recovery of spermatazoa from the vagina. Animals whose ovaries failed to show evidence of recent ovulation were excluded.

Results. Specimens from intact non-pregnant animals were studied under aerobic conditions in K.R.p. alone and with added glucose, fructose or succinate (Table I). Oxygen uptake in these media was approximately equal in the first and second hours, suggesting that tissue survival was not materially affected by the conditions of study. Oxygen uptake was unchanged by addition of glucose or fructose. It was increased 2-fold in presence of succinate. The mean lactic acid production in K.R.p. with glucose was nearly 10 times that in K.R.p. alone. This high lactic acid production was not observed with fructose or succinate. Uptake of glucose was approximately twice that of fructose.

The oviducts of 6 intact non-pregnant rabbits were studied anaerobically (Table I). Although mean glucose uptake was higher in N_2 then in O_2, the difference was not statistically significant. In K.R.p. with glucose and K.R.p. with succinate, lactic acid production was significantly higher in N_2 than it was in these media in O_2. Virtually all of the glucose taken up could be accounted for as lactic acid.

In the castrate group, oxygen uptake in the various media was not significantly different from that observed in intact animals (Table I). Glucose uptake was not significantly different in the 2 groups. Lactic acid production in glucose was significantly greater in the castrate group ($p<0.01$).

Animals studied at 16-25 hours, 43-49 hours and 64-72 hours after mating were so grouped (Table I). Oxygen uptake was not significantly different from that of intact non-pregnant rabbits. It remained essentially unchanged with time followig mating.

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TABLE I. Oxygen Uptake, Hexose Uptake and Lactic Acid Production of Oviducts Removed from Intact Animals, 23-49 Day Castrates, and Mated Animals 16 to 98 Hours after Coitus.

	Oxygen uptake, μl/mg dry wt/2 hr ± S.E.				Hexose uptake, μg/mg dry wt/2 hr ± S.E.		Lactic acid production, μg/mg dry wt/2 hr ± S.E.			
		KRp +						KRp +		
	KRp	Glucose	Fructose	Succinate	Glucose	Fructose	KRp	Glucose	Fructose	Succinate
Intact animals*	7.50 ± .63 (18)	7.74 ± .54 (18)	8.13 ± .67 (14)	16.06 ± 1.10 (20)	50.7 ± 4.3 (16)	25.3 ± 3.5 (13)	2.6 ± .5 (18)	24.9 ± 1.4 (18)	3.7 ± .4 (14)	4.3 ± .6 (20)
*Idem** (in N_2)	—	—	—	—	77.8 ± 12.8 (6)	—	9.0 ± 1.3 (6)	57.6 ± 4.6 (6)	—	9.7 ± 1.0 (6)
23-49 day castrates	5.77 ± .66 (8)	7.12 ± .22 (7)	—	16.03 ± .57 (7)	61.6 ± 3.9 (7)	—	1.4 ± .2 (7)	31.8 ± 1.3 (8)	—	3.3 ± .4 (7)
16-25 hr after mating†	7.32 ± 1.09 (4)	7.43 ± .40 (4)	—	22.38 ± 2.97 (4)	63.8 ± 14.8 (4)	—	2.5 ± .2 (4)	26.6 ± 1.8 (4)	—	7.8 ± 2.0 (4)
43-49 hr " " †	6.57 ± .50 (6)	7.57 ± .52 (7)	—	17.64 ± 1.10 (7)	54.9 ± 5.8 (6)	—	1.6 ± .4 (5)	27.9 ± 5.5 (6)	—	3.9 ± .8 (6)
64-72 hr " " †	7.42 ± .77 (4)	8.27 ± .65 (4)	—	19.33 ± 1.34 (3)	60.0 ± 12.8 (4)	—	3.4 ± .9 (4)	32.9 ± 1.8 (4)	—	6.1 ± 2.1 (4)

* Those with ovaries containing recent corpora lutea excluded.
( ) = No. of animals for which final values were obtained.
† Ovaries of all contained recent corpora lutea.

There was no significant difference in glucose uptake between mated and unmated animals. Among the mated animals, analysis of variance revealed no difference in glucose uptake with time following mating.

Lactic acid production in K.R.p. alone and K.R.p. with succinate was essentially the same for mated and unmated animals and it did not vary significantly in the interval following mating. In the presece of glucose, lactic acid production increased in the third 24-hour period after mating. This change was statistically significant by analysis of variance ($p<0.01$).

Discussion. When fresh specimens of human endosalpinx were incubated with glucose under aerobic conditions, 60% of the glucose disappearing from the medium at the end of 2 hours could be accounted for as lactic acid (1). Oxygen uptake was depressed significantly by addition of glucose to the medium. The human oviduct was stripped of serosa and muscularis, whereas the rabbit specimens in the present study included the whole oviduct.

The oviduct of the rabbit, like that of man, utilizes the anaerobic route of metabolism preferentially when incubated aerobically with glucose. Unlike the human endosalpinx, the rabbit oviduct takes up oxygen equally well in K.R.p. and K.R.p. with added glucose. When studied in nitrogen, the ability of the rabbit oviduct to utilize the anaerobic route is illustrated further.

The rate of anaerobic glycolysis becomes greater in the third day after successful mating. In this interval oxygen uptake and glucose uptake remain unchanged, while lactic acid production is increased.

The secretions of the rabbit oviduct contain rather large amounts of lactic acid(4). A significant increase in lactic acid concentration occurs during the first 3 days of pregnancy(5). Diffusion of lactic acid from the tissues of the oviduct into the tubal lumen could account for its presence in tubal fluid. The increased lactic acid production *in vitro* coincides in time with the increased concentration in tubal fluid collected continuously *in vivo*. Lactic acid is essential to the development of the 2-cell mouse ovum when cultured *in vitro*(6), and certain mammalian spermatazoa are capable of utilizing lactate for energy under aerobic conditions(7).

Summary. The oviducts of non-pregnant intact rabbits, castrates and mated rabbits 16-72 hours after coitus were studied in the Warburg apparatus. In the intact, non-pregnant group, O_2 uptake was essentially the same in Krebs-Ringer phosphate solution (K.R.p.) alone, K.R.p. with glucose and K.R.p. with fructose. It was increased 2-fold by addition of succinate. Lactic acid production was substantially higher in glucose than in fructose or in K.R.p. alone. At the end of 2 hours, glucose uptake was 50.7 ± S.E. 4.3 μg/mg dry weight of tissue and lactic acid production was 24.9 ± S.E. 1.4 μg/mg dry weight of tissue. Under anaerobic conditions virtually all of the glucose taken up could be accounted for as lactic acid. In castrates, O_2 uptake in various media and glucose uptake were not significantly different from those observed in intact, non-pregnant animals, but lactic acid output in glucose was higher. Among mated animals O_2 uptake was similar, and it did not vary significantly among specimens studied from 16-72 hours after mating. Lactic acid production in glucose was increased in the third 24-hour period following mating ($p<0.01$), indicating an increased utilization of anaerobic pathways during this interval.

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1. Mastroianni, L., Winternitz, W. W., Lowi, N. P., *Fertil. and Steril.*, 1958, v9, 500.

2. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.

3. Barker, S. B., Summerson, W. M., *ibid.*, 1941, v138, 535.

4. Bishop, D. W., *Internat. J. Fertil.*, 1957, v2, 11.

5. Mastroianni, L., Wallach, R. C., *Am. J. Physiol.*, 1961, in press.

6. Whitten, W. K., *Nature*, 1957, v179, 1081.

7. Mann, T., *Biochemistry of Semen.* London Methuen and Co., London, 1957, 53.

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Inoculation of Chicken Sarcoma Virus into Chicken Thymus. An Electron Microscopy. (26544)

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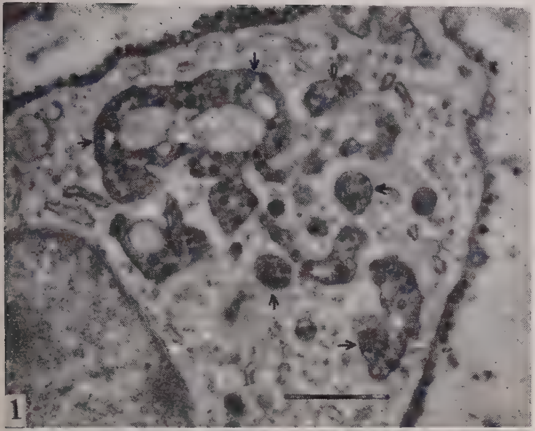
It has been reported that in the chicken sarcoma, when the tumor is transplanted subcutaneously(1,2,3,4) or intraperitoneally(5), the virus particles identifiable by electron microscope are about 50 or less per single cell section. This number has been somewhat increased by X-ray irradiation(6). Bernhard's recent report deals chiefly with extracellular viruses of Rous sarcoma(7).

We found recently that a significantly large number of virus particles was attached to the tumor cell when virus suspension was injected into the thymus.

The sarcoma of Chiba strain(8), which is 100% transplantable, was used in this experiment. A 10 g piece of tissue taken from the tumor, implanted subcutaneously 15 days before, was homogenized with a glass homogenizer in 50 cc of physiological saline and centrifuged at 4,500 rpm for 20 minutes. One ml of the supernatant virus suspension was injected into the 2nd or the 3rd thymus of 120- to 150-day-old male chickens. A tumor of myxosarcomatous pattern developed in the interlobular stroma of the thymus at site of injection, reached a macroscopic size 4 days after injection, and increased in size subsequently.

Virus particles were first detected in the tumor 4 days after injection. They were round or ovoid, 70 to 85 mμ in diameter, and consisted of a central nucleoid body, 30 to 40 mμ in diameter, surrounded with a single or double membrane (Fig. 2).



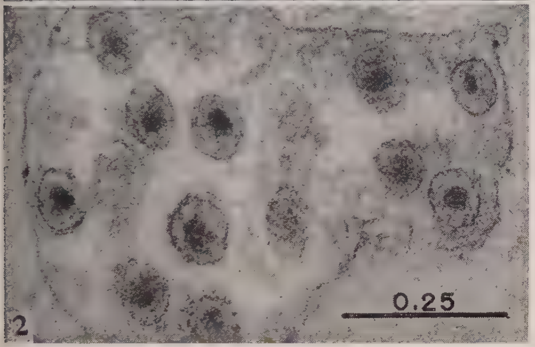


FIG. 1. Virus particles on the surface and in the peculiar intracellular spaces (arrow) of a tumor cell 6 days after intra-thymic inoculation of virus.

FIG. 2. High power view of viruses found in the peculiar intracellular space of a tumor cell. Some with double membranes. 6 days after intra-thymic inoculation.

The number of virus particles reached a maximum 6 or 7 days after inoculation, when one in 2 to 5 cells contained viruses. In some parts of the tumor, almost all tumor cells contained virus particles. Occasionally, as many as 200 to 500 virus particles were found attached to a tumor cell in a single section.

One of the conspicuous sites of virus in this period was the cell surface. Here, the virus particles were attached to the cell surface at irregular intervals or, more frequently, were arranged closely to one another in a single row (Fig. 1). Not infrequently, nearly a half or the entire circumference of the cell surface was occupied by virus particles. The virus particles on the cell surface were found embedded in a thin layer of electron dense amorphous material, which separated the particles from each other.

In the cytoplasmic vacuoles, the virus particles tended to gather in small groups. In 3 out of 6 chicks used in the winter experiment series (kept at a room temperature of 5° to 15°C and killed 6 or 7 days after inoculation), a considerable number of the particles were found in a peculiar intracellular (ergastoplasmic ?) space (Fig. 1). The virus particles were packed closely in some spaces and arranged rather loosely in others. Some of these spaces were vacuole-like and others appeared to be irregular anastomosing canaliculi. The spaces contained as a rule diffusely electron dense material in which the virus particles were embedded. A small number of viruses was present outside the cells. No virus particles were found free in the cytoplasm.

Eight days after inoculation, a sudden sharp decrease in number of virus particles detectable in the tumor was noticed.

Inoculation experiments with the same strain of virus into the subcutaneous tissue of the right wing, the testes and the kidneys showed that the number of virus particles detectable electronmicroscopically on the surface or in the cytoplasmic vacuoles of the tumor cell did not exceed 10 to 20 per single cell section. No such peculiar intracellular spaces as above described with or without virus particles were observable in tumor cells.

Although the causal factors of vigorous multiplication of Rous virus in the thymus and details of virus-to-cell relationship are subject of future studies, intra-thymic injection is worthy of trial with other tumor viruses.

Summary. Intra-thymic inoculation of virus suspension is introduced as a method for electron microscopic study of chicken sarcoma of Chiba strain. In the tumor produced in the thymus, a significantly larger number of virus particles attached to the tumor cell was observed than in those produced at other sites. The virus particles were detected chiefly on the cell surface, in cytoplasmic vacuoles and in peculiar intracellular spaces.

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1. Bernhard, W., Oberling, Ch., Vigier, Ph., *Bull. du Cancer,* 1956, v43, 407.
2. Fuse, Y., *Gann,* 1956, v47 Suppl., 530.
3. Mannweiler, Kl., Bernhard, W., *Bull. du Cancer,* 1958, v45, 223.
4. Okano, H., Nishiumi, Y., Imai, T., *Gann,* 1959, v50 Suppl., 219.
5. Epstein, M. A., *Brit. J. Cancer,* 1956, v10, 33.
6. Shimpo, K., Narimatsu, E., Nishida, K., Higashi, H., Moroya, K., *Gann,* 1959, v50 Suppl., 223.
7. Bernhard, W., *Cancer Res.,* 1960, v20, 712.
8. Chiba, T., *Gann,* 1951, v42, 2.

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“Vertical” Transmission of Passage A Leukemic Virus from Inoculated C3H Mice to their Untreated Offspring.[*] (26545)

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When the initial experiments on cell-free transmission of mouse leukemia were reported (1,2), the problem of natural transmission of the virus was also considered. It was known at that time that foster-nursing of mice born to mothers of high-leukemic strains C58(3) or Ak(4,5) by female mice of low-leukemic lines, did not prevent development of leukemia in the foster-nursed animals. Incidence of leukemia in descendants of high-leukemic strains was essentially undiminished even though such mice had been removed from the womb of their Ak mother by Caesarean section(6) and were then nursed by a low-leukemic-line foster mother. It was apparent that the leukemic agent was not transmitted through the milk, unlike the mouse mammary carcinoma virus(7). Transmission occurred most probably directly through the embryos(1).

The natural transmission of the leukemic virus from injected parents to their non-treated offspring was demonstrated in a series of experiments in which newborn C3H mice were inoculated with cell-free Ak leukemic extracts; after these mice had reached sexual maturity, they were mated and their untreated offspring observed for development of leukemia. Incidence of leukemia in the F_1 generation was significant in some experiments(2), but relatively low in others(8), since the leukemic filtrates initially employed had a low content of infective virus. After a highly potent strain of leukemic virus (passage A) was developed(9) it appeared of interest to repeat these experiments. Preliminary results of this study are reported here.

Materials and Methods. The 23rd to 26th cell-free serial passage A of the leukemic virus strain, initially derived from spontaneous Ak leukemia, was employed in this study. At this passage level, the potency of this virus is sufficiently high to induce an incidence of leukemia of over 95%, after a latency of less than 3 months, following inoculation into newborn or suckling mice of either C3H/Bi, or C57 Brown/cd inbred lines. Filtrates (Selas 02) of 20% concentration prepared in the usual manner(9) from C3H donor mice with passage A virus-induced leukemia, were inoculated intraperitoneally (0.2 to 0.3 ml). All animals were either of strain C3H(f)/Bi (free from the mammary carcinoma virus), or of the C57 Brown/cd inbred line. Incidence of spontaneous leukemia in our laboratory in mice of either strain has not exceeded, during the past several years, 0.5 to 1% in animals beyond 12 months of age.

Experimental. Development of leukemia in non-treated offspring of both virus-injected C3H(f) parents. In this series of experiments, both males and females of the C3H strain were inoculated, within a few days after birth, with passage A leukemic filtrate. After these mice reached sexual maturity, they were mated; litters were born and raised before the virus-injected parents developed, and died from, leukemia. (Several of the inoculated couples developed leukemia before they had an opportunity to raise a litter; such animals were not included in this report).

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In the first experiment (LV 398), a 3-day-old C3H(f) litter was inoculated with passage A filtrate. Three weeks later, one male and one female from this litter were mated. About 2 months later, a litter of 2 females and 3 males was born to the inoculated parents. Both virus-injected C3H(f) parents developed leukemia at 3½ months of age. All 5 non-inoculated offspring also died from leukemia at ages varying from 4 to 5 months. All leukemias were of the usually observed, lymphatic form.

In a second similar experiment (LV 399), a 3-day-old C3H(f) litter was inoculated with passage A leukemic filtrate. Three weeks later, one male and one female from the inoculated litter were mated. About 6 weeks later, a litter consisting of 4 males was born to the inoculated parents. Both virus-injected C3H(f) parents died from leukemia at the age of 3 and 4 months. Of the 4 non-injected offspring, 3 developed leukemia at 5 months, and the fourth animal at 8½ months of age.

Development of leukemia in non-treated offspring of virus-injected C3H(f) mothers, and non-inoculated C3H(f) fathers. In the first experiment of this series (LV 400), part of a C3H(f) litter was inoculated, when less than 1 day old, with the leukemic filtrate; the non-inoculated mice had their tails tied and cut, for identification. Three weeks later, one of the virus-injected females from this litter was mated to a non-injected brother. About 6 weeks later, a litter of 2 females and 3 males was born to this couple. The virus-injected mother developed leukemia at 3 months of age. Of 5 non-inoculated offspring, 3 developed leukemia when 4½ to 5 months old, and the 2 remaining mice at 6 and 8 months of age. The non-inoculated father remained healthy.

In a second experiment of the same series (LV 415), part of a 3-day-old C3H(f) litter was inoculated with leukemic passage A filtrate. Three weeks later, one of the inoculated females of this litter was mated to one of her non-injected brothers. Six weeks later, a litter of 2 males was born to this couple. The virus-injected mother developed leukemia at 3 months of age. Both non-injected offspring developed leukemia at 4½ and 5 months of age. The non-injected father remained healthy.

Observation of offspring born to non-injected mothers, and virus-injected fathers, of the C3H(f) strain. The third series of experiments was similar to the second series, except that the prospective fathers, instead of mothers, were inoculated with the leukemic virus. Three, less than 5-day-old, C3H(f) litters were split; a part of each litter was then inoculated with passage A filtrate. Three weeks later, a virus-injected male from each litter was mated to his non-injected sister.

In the first experiment of this series (LV 401), 2 successive litters, a total of 3 females and 6 males, were born to a non-inoculated mother and a virus-injected father. None of the 9 offspring has developed leukemia, although they are already over 9 months old. The virus-injected father died from leukemia at the age of 4½ months. The non-injected mother remained healthy.

In a second experiment (LV 402), 3 successive litters, a total of 5 females and 2 males, were born to a non-inoculated C3H(f) mother, and a virus-injected C3H(f) father. The inoculated father died from leukemia at 3½ months of age. None of the 7 offspring has developed leukemia, although they already are over 9 months old. The non-injected mother also remained healthy.

In a third experiment of this series (LV 403), 4 successive litters, a total of 5 females and 10 males, were born to a non-inoculated C3H(f) mother, and a virus-injected C3H(f) father. None of the 15 offspring has developed leukemia, although they are now more than 9 months old. The virus-injected father died from leukemia at 4 months of age. The non-injected mother remained healthy.

Failure of offspring of virus-injected C57 Brown/cd parents to develop leukemia. Newborn, suckling, and young adult mice of the C57 Brown/cd strain are as susceptible to the leukemogenic action of the passage A virus as are mice of the C3H/Bi strain(10,11). It was therefore of interest to determine whether in mice of this strain also, offspring of inoculated parents would develop leukemia. Newborn and suckling, less than 7-day-old, C57 Brown/cd litters were inoculated with passage A filtrate. After the inoculated mice reached sexual maturity, they were mated to their own littermates. In the first 2 experiments, both parents were inoculated. A litter of 3 mice was born to the first couple, and a litter of 2 mice to the second couple. Only 1 developed leukemia at 7½ months of age; the remaining 4 mice are still in good health, although they are now over 9 months old. All 4 inoculated parents died from leukemia at 3½ to 4½ months of age.

In a second group, consisting of 2 couples, a total of 19 offspring (11 females and 8 males) was born to virus-injected C57 Brown/cd mothers, and non-injected C57 Brown/cd fathers. None of the 19 offspring (4 litters) has developed leukemia, although they are now more than 9 months old. Both virus-injected C57 Brown/cd mothers died from leukemia at 4 and 4½ months of age. The non-injected fathers remained healthy.

In an additional series consisting of 3 C57 Brown/cd couples, non-injected C57 Brown/cd females were mated to their virus-injected brothers. None of the 40 offspring (6 litters) born to such parents has thus far developed leukemia; they are now more than 9 months old. All 3 virus-injected C57 Brown/cd fathers developed leukemia at 3 to 3½ months of age. The 3 non-injected C57 Brown/cd mothers remained healthy.

Discussion. The high incidence of leukemia in successive generations of mice of certain inbred strains, such as C58 or Ak was at first explained by genetic factors(3,12). Transmission of a leukemic factor through milk of nursing females to their offspring was considered, but was promptly excluded by foster-nursing experiments(3,4,5).

When the viral nature of mouse leukemia was demonstrated(1,2), natural transmission of the leukemic agent from one generation to another was again considered. Development of leukemia in offspring of high-leukemic inbred strains, such as Ak, could not be prevented even if the embryos were removed at term by Caesarean section from the womb of their Ak mother, and transmitted for foster nursing to female mice of a low-leukemic strain(8); moreover, Ak mice born from transferred Ak ova also developed leukemia (13). That the leukemic virus is present in the developing embryo was demonstrated in direct bio-assay experiments in which extracts prepared from normal, healthy, Ak(1, 2,8), or C58(14), embryos, induced leukemia following inoculation into newborn C3H mice.

The virus is probably transmitted from parents to offspring directly through the fertilized ovum; it is possible, however, that the virus could pass through the placenta in the pregnant, virus-carrying, female, and infect the embryo.

The term "vertical transmission" was suggested to designate the natural passage of a pathogenic virus from one generation to another(2). Actual transmission of the virus from virus-injected parents to their offspring could be demonstrated. When newborn mice of strain C3H were inoculated with Ak leukemic extracts, and when such virus-injected mice were subsequently mated, some of their non-treated offspring developed leukemia(1, 2,8). However, in previous studies, a leukemic virus of low potency was employed, and probably for that reason incidence of leukemia developing in offspring of the inoculated C3H parents was relatively low; furthermore, the disease usually appeared late. In the present study, a considerably more potent leukemic virus was employed for inoculation of the prospective parents. Incidence of leukemia in the offspring of the inoculated parents was high, and latency period relatively short.

Curiously, inoculation with the leukemic virus of the prospective mothers only, had in our present experiments as much influence on incidence of leukemia developing in their offspring, as had the inoculation with the virus of both prospective parents. When the prospective fathers only were inoculated, and later mated to non-injected females, no leukemia has appeared in their offspring; additional observations are needed, nevertheless, to clarify this point. The animals in this series, now only about 9 months old, should be observed for a longer period. In our previous similar studies (Gross, unpublished) we have observed occasional development of leukemia in offspring of non-injected mothers and virus-injected fathers of the C3H strain; however, incidence of leukemia in such offspring was low, and latency prolonged. It was apparent nevertheless, that under proper experimental conditions the leukemic virus could be transmitted from one generation to another not necessarily through the mother, but also through a virus-carrying father.

It has been observed(3,15,16) that incidence of leukemia in F_1 generation of hybrids was higher, at least in some experiments, when the mother was of the high-leukemic-line, such as Ak or C58, and the father of a low-leukemic strain, such as C3H, Rf, or StoLi. If the experimental conditions were reversed, *i.e.*, father was of the high-leukemic strain, and the mother of a low-leukemic line, the F_1 generation of hybrids had a lower incidence of leukemia. In other experiments, however, incidence of leukemia in F_1 generation of hybrids was approximately similar when either the mother or the father were of a high-leukemic strain(3,12,15,16).

Mice of the C57 Brown/cd strain are highly susceptible to the leukemogenic action of passage A virus. When suckling C57 Brown/cd mice were inoculated with the passage A virus, practically 100% of them developed leukemia after a latency of about 3 months(11; also unpublished); yet most of the offspring of virus-injected C57 Brown/cd mice did not develop leukemia in our experiments, even when both parents had been inoculated with the passage A virus. Offspring of such virus-injected C57 Brown/cd mice, still in good health, are now more than 9 months old; additional observations are needed to determine whether some of them will not eventually develop leukemia. It appears, however, that mice of the C57 Brown/cd strain, though highly susceptible to the leukemogenic action of the passage A virus, do not transmit this virus to their offspring as readily as do mice of the C3H strain. Susceptibility to the oncogenic action of a virus is thus distinct from the ability of the host to transmit the inoculated virus naturally to its offspring.

Summary. 1. C3H(f) mice inoculated within a few days after birth with passage A leukemic filtrates, were subsequently mated. 2. When both females and males selected for mating received inoculation of the passage A virus, or when inoculated females were mated to non-inoculated males, all offspring born to such parents developed leukemia at 4½ to 7 months of age. 3. When, however, non-inoculated females were mated to virus-injected males, 31 non-treated offspring have remained thus far in good health, although they have already reached 8 to 9 months of age. 4. Similar experiments were performed employing mice of the C57 Brown/cd strain, highly susceptible to leukemogenic action of passage A virus. 5. Of the 64 non-treated C57 Brown/cd offspring, now 9 months old, only 1 has thus far developed leukemia when either a) both parents, b) mothers only, or c) fathers only had been injected with the passage A virus.

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1. Gross, L., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 27.

2. ———, *ibid.*, 1951, v78, 342.

3. MacDowell, E. C., Richter, M. N., *Arch. Path.*, 1935, v20, 709.

4. Barnes, W. A., Cole, R. K., *Cancer Research*, 1941, v1, 99.

5. Furth, J., Cole, R. K., Boon, M. C., *ibid.*, 1942, v2, 280.

6. Gross, L., *Ann. N. Y. Acad. Sci.*, 1952, v54, 1184.

7. Bittner, J. J., *Science*, 1936, v84, 162.

8. Gross, L., *Acta Haemat.*, 1955, v13, 13.

9. ———, PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 767.

10. ———, *ibid.*, 1954, v86, 734.

11. ———, *Acta Haemat.*, 1960, v23, 259.

12. Furth, J., Seibold, H. R., Rathbone, R. R., *Am. J. Cancer*, 1933, v19, 521.

13. Fekete, E., Otis, H. K., *Cancer Research*, 1954, v14, 445.

14. Gross, L., *Cancer*, 1956, v9, 778.

15. Cole, R. K., Furth, J., *Cancer Research*, 1941, v1, 957.

16. Law, L. W., *Ann. N. Y. Acad. Sci.*, 1954, v57, 575.

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Intracellular Distribution of Trace Elements in Liver Tissue.* (26546)

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Determinations of metals in whole liver tissue have revealed a general pattern of concentrations(1-3). Attempts to correlate abnormal conditions and changes in trace element concentration have shown promise, but the variations among samples require a detailed statistical study of large numbers of organs(4-10). The present study is an attempt to determine the intracellular distribution of selected metals found in liver tissue fractionated by differential centrifugation. Knowledge of the intracellular location of a trace element is of value in determining its function and alterations in that distribution might be characteristic of pathological states.

Methods. The usual precautions applicable to trace element analyses to minimize contamination were observed. Sucrose (0.25 M) was passed twice through an IR 120 Amberlite resin column to remove metallic impurities. Qualitative spectrographic examination for the elements of interest revealed only a trace of copper. Blank determinations were run concurrently with samples. The wet ashing technic and spectrographic method employed was a modification of those reported elsewhere(11). Nitrogen was determined in an aliquot of the samples after the method of Ma and Zuazaga(12).

Animals were acclimatized for 2 weeks in similar, individual cages and fed Purina Lab Chow from a single batch. Human liver specimens were secured in the operating room and immediately fractionated. There was no trace element evaluation of diets or medications received by the patients.

Procedure. Five groups of 5 normal young male rats of Wistar strain were used to determine mean values. The fifth group was also a control for 5 rats into which Walker rat carcinosarcoma 256 had been transplanted. Animals were killed by decapitation, livers were removed immediately and rinsed with cold, 0.25 M Sucrose. All subsequent operations were performed at a temperature of 0-5°C. Livers were finely minced in the medium with stainless steel scissors and homogenized with a plastic and glass Potter-Elvehjem type homogenizer. An aliquot was taken for analysis as the homogenate sample.

The fractionation procedure followed was essentially that of Hogeboom, Schneider and Pallade(13). The homogenate was centrifuged in a Spinco refrigerated centrifuge for 10 minutes at 500 × g and separated by decantation. The sediment was rehomogenized with 4-5 volumes of sucrose and again centrifuged for 10 minutes at 500 × g. The supernatants were combined. The sediment was collected with distilled water and frozen for later analysis, as the "nuclear" fraction. This fraction also contained some unbroken cells, blood cells, connective tissue and mitochondria in addition to free nuclei.

The combined supernatants from the first step were centrifuged for 10 minutes at 7,000 × g, decanted, and sediment washed with sucrose, collected and analyzed as the "mitochondria" fraction.

The remaining supernatant portion was further centrifuged for 60 minutes at 105,000 × g and sediment collected as the "microsome" fraction.

For analysis, frozen samples were thawed, homogenized and aliquots taken for nitrogen determination. The principle portions of samples were wet ashed in covered beakers with the exception of supernatant samples. The large amount of sucrose in supernatant samples made wet ashing of this fraction impractical. Instead, samples were made acid ($pH<1$) with concentrated hydrochloric acid, precipitated proteins filtered off and metals precipitated directly from the filtrate. The ash from the protein precipitate was examined spectrographically and found to be

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TABLE I. Distribution of Trace Elements in Normal Rat Liver Fractions. Average results of 5 groups with standard errors of means.

	Results expressed as % of reconstituted total						
	N	Fe	Zn	Cu	Mo	Mn	Cr
Nuclei	37 ± .6*	37 ± 4.9	31 ± 3.6	30 ± 7.2	28 ± 8.1	37 ± 4.9	49 ± 9.9
Mitochondria	18 ± .6	10.7 ± 1.2	9.3 ± 1.3	17 ± 3.0	38 ± 5.3	39 ± 3.8	14 ± 3.0
Microsomes	17 ± .4	21 ± 1.6	10.6 ± .6	4.8 ± .8	6.1 ± 1.4	14 ± 2.2	14 ± 2.6
Supernatant	28 ± .7	32 ± 7.2	49 ± 4.8	48 ± 8.3	27 ± 7.1	9.7 ± 2.8	23 ±10

	Results expressed as μg of element per mg of nitrogen					
	Fe	Zn	Cu	Mo	Mn	Cr
Nuclei	2.6 ± .27 *	.77 ± .062	.25 ± .12	.011 ± .0006	.029 ± .0079	.0041 ± .00090
Mitochondria	1.7 ± .071	.42 ± .030	.19 ± .046	.025 ± .0024	.067 ± .031	.0029 ± .00076
Microsomes	3.6 ± .83	.65 ± .087	.061 ± .020	.0050 ± .00036	.032 ± .017	.0026 ± .00047
Supernatant	5.0 ±2.9	2.0 ± .43	.39 ± .065	.019 ± .00061	.0072 ± .0034	.0040 ± .0019
Reconstituted total	3.3 ± .84	1.05 ± .17	.25 ± .040	.015 ± .0009	.037 ± .015	.0036 ± .00024

* ± S.E.

free from detectable amounts of metals of interest.

Results. Mean values for normal rat liver fractions are presented in Table I and include standard errors obtained for the 5 groups of normal rat livers. Values for nitrogen and metals present in determinable quantities are shown both in terms of percent of element in the "reconstituted total" and as micrograms of element per milligram of nitrogen. Metals sought but not found in determinable quantities were Ag, Co, Ni, Pb, Sn, Ti, and V.

Distribution of the metals studied is reproducible and consistent with similar work reported by others(14).

When compared to nitrogen, the "nuclear" fraction is at or near whole tissue level for all of the elements determined with the possible exception of chromium which appears to be concentrated in this fraction. The heterogeneity of this fraction may account in part for this similarity to whole tissue values.

The mitochondrial fraction contains molybdenum and manganese in greater than whole tissue concentration when compared to nitrogen. Copper occurs in approximately equal ratio, and the other elements in less than whole tissue values. Several enzymes have been found to be concentrated in this fraction(15-17). Since the enzymes known to contain molybdenum have been reported to be found mostly in the supernatant fraction (20), the concentration of this element in the mitochodrial fraction suggests the possibility of other molybdenum containing enzymes.

The microsomal fraction contains less than whole tissue concentrations of the metals studied except iron which accounts for the pigmentation of this fraction(18).

The supernatant fluid contains iron, zinc, copper and molybdenum in concentrations greater than whole liver tissue when compared to nitrogen. Many of the metal containing enzymes have been found to be present in this fraction(17,19,20) and the enrichment of these metals would be expected.

Comparison of trace element content of normal and tumor bearing animals. Two previous experiments on large groups of animals with transplanted Walker rat carcinosarcoma 256 tumor disclosed no discernible differences of trace element content in the livers of control and tumor-bearing rats† To determine whether the trace element distribution pattern in rat liver cell fractions might be altered even though whole liver values were unchanged, the tumor was transplanted into the axilla of 5 rats and liver cell fractionation performed after the tumors had reached considerable size about 7 days later. Table II presents the element nitrogen ratios of treated and control animals. No differences in distribution of the metals determined are revealed by comparing the 2 groups.

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† Personal data accumulated in U.S.P.H.S. Progress Reports (C-1121).

TABLE II. Distribution of Trace Elements in Rat Liver Fractions. Results expressed as μg of element per mg of nitrogen.

Avg results of 5 normal control animals

	Fe	Zn	Cu	Mo	Mn	Cr
Nuclei	3.1	.86	.063	.010	.044	.0024
Mitochondria	1.8	.48	.11	.021	.11	.0015
Microsomes	5.0	.81	.025	.0046	.065	.0034
Supernatant	10.6	2.9	.52	.031	.014	.0079
Reconstituted whole liver	5.3	1.4	.20	.017	.051	.0040

Total liver sample, 58.0 g

Avg results of 5 tumor-bearing animals

	Fe	Zn	Cu	Mo	Mn	Cr
Nuclei	3.2	.64	.071	.0088	.046	.0034
Mitochondria	1.8	.44	.13	.019	.13	.018
Microsomes	4.8	.71	.024	.0052	.026	.0031
Supernatant	8.0	2.8	.43	.023	.015	.0068
Reconstituted whole liver	4.5	1.2	.20	.014	.049	.0067

Total liver sample, 54.5 g

In comparing the controls in Table II with 5 groups of control animals in Table I, there is good agreement of values except for manganese in the supernatant fractions and manganese and copper in the microsomes. These apparent differences are believed to represent variations among groups of normal animals as shown in the standard error values in Table I.

Determinations of trace elements in human liver has revealed a wide range of values with different conditions(8). Fractionation of 2 specimens of human liver obtained at surgical operations demonstrate a difference in intracellular distribution as well as in total trace element concentration. The small sample size of one specimen casts some doubt on the reliability of the results. Table III presents values obtained in a human liver fraction obtained at surgery from a patient with carcinoma of the colon. The liver was grossly and microscopically free of disease, but a significant anemia was present prior to operation and undoubtedly accounts for the marked decrease of iron in all fractions. Table IV indicates intracellular distribution of trace metals in a patient with chronic cholecystitis. This liver was histologically normal. There are apparent differences which could reflect disease processes. The paucity of specimens as well as sample size prevents any conclusions, but the results suggest that cellular fractions may reveal more trace element abnormalities than are evident in whole liver analyses.

TABLE III. Distribution of Trace Elements in Human Liver Fractions. Diagnosis: Carcinoma of colon (liver grossly normal).

Results expressed as % of reconstituted total

	N	Fe	Zn	Cu	Mo	Mn	Ni	Sn
Nuclei	17	37	25	69	8.1	30	56	62
Mitochondria	19	21	5.2	9.4	27	36	8.8	15
Microsomes	21	21	6.0	6.9	23	14	12	7.0
Supernatant	43	20	64	15	42	20	23	15

Sample size, 4.1 g

Results expressed as μg of element/mg of nitrogen

	Fe	Zn	Cu	Mo	Mn	Ni	Sn
Nuclei	3.6	3.9	6.7	.011	.091	.39	.091
Mitochondria	1.8	.70	.81	.032	.097	.054	.020
Microsomes	1.6	.71	.52	.024	.034	.067	.0081
Supernatant	.76	3.8	.57	.022	.024	.062	.0088
Rec'nstit'd whole liver	1.6	2.6	1.6	.023	.051	.12	.025

TABLE IV. Distribution of Trace Elements in Human Liver Fractions. Diagnosis: Chronic cholecystitis.

Results expressed as % of reconstituted total

	N	Fe	Zn	Cu	Mo	Mn	Cr	Ni
Nuclei	31	30	25	34	26	24	50	81
Mitochondria	11	9.7	5.3	7.4	14	25	10	4.7
Microsomes	16	18	8.8	4.4	11	8.7	12	0
Supernatant	42	42	61	54	49	43	28	14

Sample size, 24 g

Results expressed as μg of element/mg of nitrogen

	Fe	Zn	Cu	Mo	Mn	Cr	Ni
Nuclei	46	2.5	.57	.011	.038	.065	.12
Mitochondria	41	1.5	.34	.017	.11	.036	.020
Supernatant	48	4.5	.68	.017	.051	.027	.016
Reconstituted whole liver	47	3.1	.52	.014	.050	.040	.047

Summary. Intracellular distribution of 6 metals in liver tissue has been determined for 5 groups of normal rats. Introduction of the Walker rat carcinosarcoma 256 did not change the total level or distribution pattern for the metals determined between treated

and control rats. Two specimens of human liver tissue were fractionated and trace metal distribution determined.

1. Butt, E. M., Nusbaum, R. E., Gilmore, T. C., Didio, S. L., *Am. J. Clin. Path.*, 1954, v24, 385.

2. Koch, H. J., Jr., Smith, E. R., Shimp, N. F., Connor, J., *Cancer*, 1956, v9, 499.

3. Tipton, I. H., Cook, M. J., Steiner, R. L., Foland, J. M., McDaniel, K. K., Fentress, S. D., ORNL No. 57-2-4, Oak Ridge Nat. Lab. (1957) Mimeo.

4. Addink, N. W. H., *Nature*, 1950, v166, 693.

5. Bertrand, G., Vladesco, R., *Compt. Rend. Acad. Sci.*, 1921, v173, 176.

6. Chabovitch, X., Kachanin, B., *Glas. Srpske Akad. nauk. Belgrade*, 1955, v215, 147.

7. Heggen, G. H., Olson, K. B., Edwards, C. F., Clark, L. B., Maisel, M., *Radiation Research*, 1958, v9, 285.

8. Olson, K. B., Heggen, G. H., Edwards, C. F., *Cancer*, 1958, v2, 554.

9. Sandberg, M., Gross, H., Holly, O. M., *Arch. Path.*, 1942, v33, 834.

10. Vallee, B. L., *J.A.M.A.*, 1956, v162, 1053.

11. Heggen, G. E., Strock, L. W., *Anal. Chem.*, 1953, v25, 859.

12. Ma, T. S., Zuazaga, G., *Ind. Eng. Chem.*, Anal. Ed., 1942, v14, 280.

13. Hogeboom, G. H., Schneider, W. C., Palade, G. E., *J. Biol. Chem.*, 1948, v172, 619.

14. Thiers, R. E., Vallee, B. L., *ibid.*, 1957, v226, 911.

15. DeDuve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., Appezmans, F., *Biochem. J.*, 1955, v60, 604.

16. Hogeboom, G. H., Claude, A., Hotchkiss, R. D., *J. Biol. Chem.*, 1946, v165, 615.

17. Hogeboom, G. H., Schneider, W. C., Striebich, M. J., *Cancer Research*, 1953, v13, 617.

18. Strittmatter, C. F., Ball, E. G., *Proc. Nat. Acad. Sci.*, 1952, 38, 19.

19. Anderson, N. G., *Exp. Cell Res.*, 1956, v11, 186.

20. Villela, G. C., Mitidieri, E., Affonso, O. R., *Nature*, 1955, 175, 1087.

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Patterns of Increase and Spread of Polioviruses in Monkeys after Inoculation into Traumatized Tissue.* (26547)

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If poliomyelitis virus in adequate concentration is inoculated into healing skin wounds of cynomolgus monkeys, an increase of virus in the local site and in other parts of the body follows(1). Data are presented here to show the patterns of increase and spread of 4 different strains of type 1 virus chosen principally for characteristic virulence as measured experimentally or indicated epidemiologically.

Virus strains. Each of the 4 strains of virus used in this study had distinct characteristics of especial interest. Two strains, Mahoney and LSc, had been modified during prolonged passage in tissue culture in other laboratories. The other 2, Chesterfield Inlet and Iberia, were essentially "field" strains; that is, they had undergone only 2 passages in the laboratory. All were identified as type 1 poliovirus by neutralization with homotypic antiserum and by the fact that monkeys infected with each strain developed antibodies to type 1 poliovirus of the Mahoney strain.

The Chesterfield Inlet strain was obtained from feces of a patient who developed poliomyelitis during the epidemic in this arctic community in 1949. This was one of the world's most violent epidemics(2). In a total population of 275 Eskimos living in the village at that time, 18.5% were paralyzed and 5% died. The feces were collected April 27, 1949, and were sent to us by Dr. F. P. Nagler. They were preserved 7 years in the frozen state prior to use. Virus was then freshly isolated in monkey kidney tissue cultures, and second passage material was used for inoculation of monkeys in the present experiments.

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The Iberia virus was received from Drs. John Fox and Henry Gelfand in the form of feces from a child living in Iberia Parish, Louisiana. The specimen was obtained on June 1, 1955, as a routine collection during a family study of Louisiana communities(3). The child had no illness at that time; clinical poliomyelitis was not prevalent in the community; and the attack rate was low, 2.4/100,000 population, for the entire parish in 1955. Virus of the second passage in tissue cultures of monkey kidney was employed.

The Mahoney strain of virus has been studied extensively and is included in the killed virus vaccine distributed commercially in the United States. For our experiments virus received from the Connaught Laboratories in 1954 was used. This was in fluid from infected cultures of monkey renal tissues and was preserved by freezing.

Dr. Albert B. Sabin provided us with the LSc strain of virus in 1956. It was derived from the Mahoney strain by Drs. Li and Schaeffer in 1954(4) and has been purified by 3 successive single plaque isolations by Dr. Sabin(5). It is notably avirulent for monkeys and man. The virus sent to us was a portion of the culture as seed for Dr. Sabin's type 1 vaccine.

Repeated tests in monkey kidney tissue tube cultures inoculated with 0.1 ml of suitably diluted stock virus showed titers satisfactorily reproducible, expressed as $TCID_{50}$ per ml, at the following levels: Chesterfield Inlet $10^{7.5}$, Iberia $10^{7.2}$, Mahoney $10^{6.8}$, LSc $10^{7.6}$.

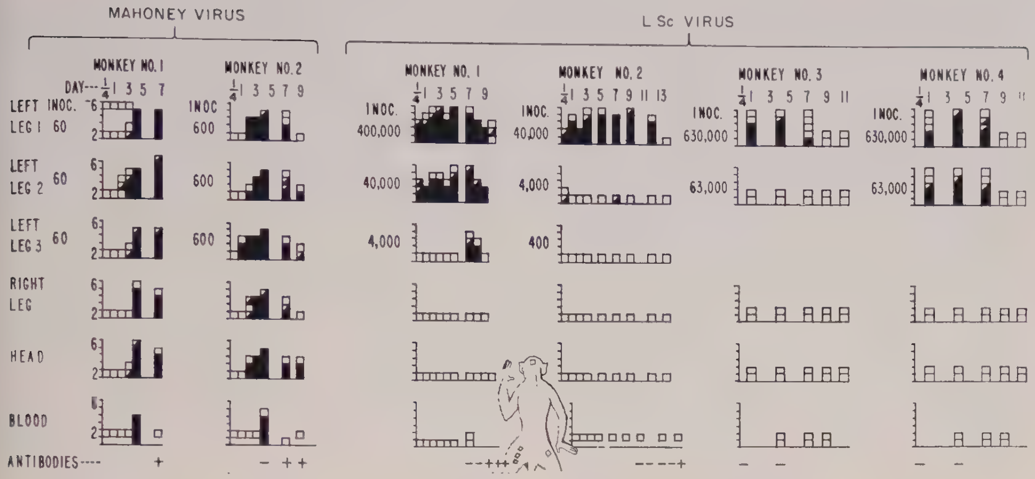
Methods. Procedures employed were essentially similar to those described previously (1). Tissue cultures were prepared from trypsinized monkey (rhesus or cynomolgus) renal tissue. Cells were grown directly on glass in test tubes and were stored for periods of 1 to 6 weeks as previously described. Titrations were based on the inoculation of appropriately diluted specimens into duplicate tube cultures.

Monkeys were obtained from several sources. Excision of 1 square cm of skin was carried out 3 days before inoculation of virus. Procaine penicillin and streptomycin were used to prevent infection, and lesions did not show signs of bacterial invasion. They remained clean and healed at the expected rate. In fact, by the 14th day after inoculation epithelium covered the lesion and had to be abraded to get material for testing.

Five skin lesions were created on each monkey, 1 on the scalp, 1 on the lateral aspect of 1 thigh, usually the right, and 3 in a vertical row on the lateral aspect of the other thigh. Adjacent lesions were separated by 1.5 cm of normal skin (Fig. 1). Three days after excision of skin, the 3 lesions on 1 thigh were inoculated with virus. The lesions on the opposite thigh and the lesion on the scalp were left uninoculated.

After inoculation the monkeys were restrained in a box similar to that described by Ives and Dack(6) or in a "monkey chair" to avoid external transfer of virus from inoculated lesions to those not inoculated. Monkeys were fed by an attendant while restrained. Their food intake was, in many cases, greatly reduced during this time.

In "harvesting" exudate from lesions the monkey was etherized, skin around the lesion was cleansed with anesthetic ether, and the scab was removed with sterile forceps. Then, with a second pair of sterile forceps, a ball of cotton approximately 3 mm in diameter was pressed into all parts of the raw surface to absorb the serous fluid that oozed from the base of the lesion. Little bleeding was ordinarily encountered after the first 3 or 4 days. The cotton was then placed in 1 ml of chilled "medium E" (mixture 199 with 100 units of penicillin, 100 μg of streptomycin, and 25 units mycostatin per ml), to which 0.2 ml of anesthetic ether had been added. The specimen was agitated to mix the ether with the aqueous phase and stored at 4°C. Titration of virus was usually carried out the next day. Specimens collected on Friday (usually the 4th day after inoculation of the monkey) were ordinarily titrated the following Monday. In preliminary experiments biopsy of the base of the lesion was shown to yield no higher concentration of virus than was found in the exudate collected as just described. The amount of serous exudate collected was



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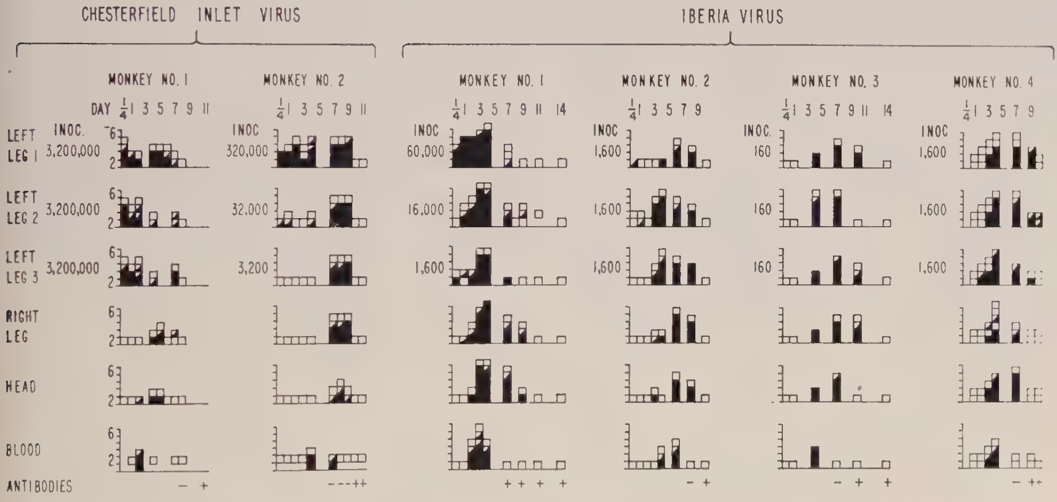

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FIG. 1 and 2. Changes in concentration and distribution of polioviruses after inoculation into skin wounds of cynomolgus monkeys. One cm^2 of skin was removed from sites shown on the diagram in Fig. 1. The 3 lesions on the lateral aspect of 1 thigh, usually the left, are designated as Left Leg 1, 2, and 3 to indicate proximal, middle, and distal lesions. (Monkeys 3 and 4, LSc virus, had only 2 lesions instead of 3.) "Inoc" denotes amount of virus injected, expressed in tissue culture ID_{50}. Numbers on vertical axis represent the negative logarithm of the dilution of exudate or blood tested for virus. Dark areas represent presence of virus as follows: ■ Test for virus was positive in all tissue cultures, usually 2, more if test was repeated. □ Test for virus was negative in all cultures, usually 2, more if test was repeated. ◪ Test for virus was positive in 1 or more cultures and negative in 1 or more cultures. Signs of illness were as follows:

Mahoney Virus: Monkey No. 1—Paralysis first observed on 7th day. Animal died the same day. Monkey No. 2—Tremors at 7 days, paralysis at 9 days. Died on 11th day.

LSc Virus: No illness detected in any of the 4 monkeys.

Chesterfield Inlet Virus: Monkey No. 1—Tremors observed at 8 days. No definite paralysis. Monkey No. 2—Tremors at 11 days, definite paralysis at 13 days.

Iberia Virus: Monkey No. 1, 2, and 3—No illness detected. Monkey No. 4—Paralysis observed at 10 days.

approximately 10 mg. Therefore, the specimens as collected in 1 ml of diluent were regarded as 10^{-2} dilutions of exudate.

Blood was collected from a superficial vein distal to the popliteal area on the leg with the uninoculated skin lesion. One-tenth to 0.2 ml were placed in 1 ml of chilled medium E. The specimen was refrigerated, and a portion was inoculated into tissue cultures at the same time as the other specimens harvested on that day. Tests for antibody were made by removing red cells and mixing 0.27 ml of the supernatant fluid with 0.03 ml of an appropriate dilution of Mahoney virus. After 30 minutes' incubation at 23°C tissue cultures were inoculated with 0.1 ml of this mixture representing approximately 100 ID_{50} of virus and 10% serum.

Histopathologic examination of the spinal cord of monkeys that showed paralysis confirmed the diagnosis of poliomyelitis. Those that showed no paralysis were examined daily in the cage for 1 month.

Results. In Fig. 1 and 2 the results of assays of virus during the course of infections are presented. *Minimal infective dose.* The minimal infective dose of LSc virus appeared to be approximately 4,000 $TCID_{50}$; however, 63,000 $TCID_{50}$ failed to cause infection of 1 lesion. Infection resulted from injection of 160 $TCID_{50}$ of Iberia virus and 60 $TCID_{50}$ of Mahoney virus. In additional experiments not included in the figures, no infection resulted from inoculation of approximately 16 $TCID_{50}$ of Iberia virus into 3 skin lesions of 1 monkey nor from injection of approximately 6 $TCID_{50}$ of Mahoney virus into skin wounds of each of 2 monkeys.

Pattern of viral increase and decrease in inoculated and uninoculated lesion. The LSc virus, unlike the other 3 strains, failed to spread from inoculated skin lesions to uninoculated lesions. In 2 monkeys it even failed to infect a lesion only 1.5 cm from an infected lesion.

In animals inoculated with infecting concentrations of the other 3 strains, virus increased in the injected lesion and invariably appeared in uninoculated lesions on the scalp and opposite thigh. Concentration of virus in exudate was relatively high and approximately equal in inoculated and uninoculated lesions. A pattern of delayed viral increase in obviously infected lesions occurred in several instances (see Mahoney Monkey 1 and Iberia Monkey 2 for example). In left leg lesion 2 of LSc Monkey No. 2, virus was present at 6 hours and 7 days at minimal detectable levels but did not increase.

In some animals virus increased first in inoculated lesions. Chesterfield Inlet Monkey No. 2 is the most striking example of this phenomenon. In most animals rise of virus titer occurred almost simultaneously in all 5 lesions, inoculated and uninoculated.

Decline of virus titer as a rule progressed over a 24- or 48-hour period. However, decrease of virus occurred precipitously in all 5 lesions in one monkey, Chesterfield Inlet Monkey No. 2. Although there was some difference from one lesion to another, the general pattern of viral decline was that of a systemic immunity manifest in all 5 skin sites simultaneously and to an essentially equal extent.

Neuropathogenicity. Paralysis was seen in both monkeys injected with Mahoney virus, in 1 of the 2 injected with Chesterfield Inlet virus, and in 1 of the 4 animals inoculated with Iberia virus. The lack of virulence of the LSc strain is consistent with many other studies of this strain.

In all 4 monkeys that became paralyzed neurological damage appeared and progressed at the same time that virus in skin lesions was declining, presumably in response to antibody as demonstrated in the serum. Monkey No. 2, given Chesterfield Inlet virus, demonstrates this relationship clearly. Virus concentration was maximal in all skin lesions of this animal at 8 and 9 days. At 10 days virus had disappeared from all 5 skin lesions. Yet paralysis was not detected at 10 or 11 days but was definite at 13 days. Antibodies were demonstrated at 10 and 11 days.

Discussion. Wenner and Kamitsuka(7) have stressed the importance of recognizing that sites of implantation of virus and sites of replication are not synonymous. They showed that Brunhilde virus inoculated intramuscularly multiplied first in inoculated muscles(8). Virus in lymph nodes of their animals did not exceed the amount that could result from implantation. In the present experiments maximal concentration of virus in most lesions far exceeded that found in any specimen of blood. Therefore, it seems clear that virus multiplied in tissues of these areas, whether inoculated directly or infected as a result of spread within the monkey.

The term "infection of skin lesions" in the present work should not be construed to mean infection of skin. The base of the lesion was loose connective tissue altered by the trauma of skin excision and by the healing process. Virus assays were performed on fluid exuding from this tissue. Which cells were infected and whether comparable cells in uninjured tissue of normal animals are susceptible is unknown.

In previous studies a discrepancy has been noted between capacity of poliovirus to grow in testicular and renal tissue *in vitro* and inability to grow in these tissues in the animal (9, 10). The free multiplication of poliovirus in the base of healing skin wounds raises the question of whether the tissues involved are altered by the response to trauma in a manner that makes them physiologically similar to cells in tissue culture. If this is true, it may be a helpful clue in the search for factors that determine the extent of extraneural growth of poliovirus and the likelihood of invasion of the nervous system.

With the Mahoney, Chesterfield Inlet, and Iberia viruses there was no indication that an uninoculated lesion on the opposite thigh was infected earlier or later than one on the scalp. The differences were relatively slight. This finding favors the concept of hematogenous spread, as spread *via* neural pathways might be expected to infect a lesion on the opposite thigh before one on the head.

The procedure used in these studies appears to provide a convenient method of determining the capacity of poliovirus strains to multiply in extraneural sites outside of the alimentary canal and to spread throughout the body by the hematogenous route. This experimental system may be a potentially useful tool in analysis of viruses proposed for use in oral vaccines.

Summary. Four strains of type 1 poliovirus were injected into loose connective tissue at the base of lesions created 3 days previously by excision of 1 cm^2 of skin of one thigh. Viral content of serous exudate from inoculated lesions and from uninoculated skin lesions on the scalp and the opposite thigh was determined. The Chesterfield Inlet and Iberia viruses were chosen as field strains that were epidemiologically virulent and avirulent respectively. The Mahoney and LSc are well known laboratory strains of high and low virulence. Less than 200 TCID$_{50}$ of Mahoney and Iberia viruses were required to infect these lesions. Infection occurred irregularly with LSc virus injected in amounts of 4,000 and 60,000 TCID$_{50}$. The avirulent LSc virus failed to appear in the blood or in uninoculated lesions. The other 3 strains increased in inoculated lesions and in all uninoculated lesions. Although virus appeared to multiply extensively at the lesion site, healing progressed normally; and there was no evidence of local tissue injury. The decrease and disappearance of virus in all lesions of any one animal was essentially parallel. Paralysis occurred in the 2 monkeys infected with Mahoney virus, in 1 of 2 infected with the Chesterfield Inlet virus, in 1 of 4 infected with Iberia virus, and in none of the 4 infected with LSc virus.

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1. Evans, C. A., Hoshiwara, I., Proc. Soc. Exp. Biol. and Med., 1955, v89, 86.

2. Peart, A. F. W., *Can. J. Pub. Health,* 1949, v40, 405.

3. Fox, J. P., Gelfand, H. M., LeBlanc, D. R., Conwell, D. P., *Am. J. Hyg.,* 1957, v65, 344.

4. Li, C. P., Schaeffer, M., Proc. Soc. Exp. Biol. and Med., 1954, v87, 148.

5. Sabin, A. B., *J. Am. Med. Assn.,* 1957, v164, 1216.

6. Ives, M., Dack, G. M., *J. Lab. and Clin. Med.,* 1956, v47, 723.

7. Wenner, H. A., Kamitsuka, P., *Virology,* 1956, v2, 83.

8. ———, *ibid.,* 1957, v3, 429.

9. Evans, C. A., Byatt, P. H., Chambers, V. C., Smith, W. M., *J. Immunol.,* 1954, v72, 341.

10. Kaplan, A. S., *Virology,* 1955, v1, 377.

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Lipoprotein Content of Human Pathological Serous Fluids.* (26548)

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The enigma of lipid transport in the aqueous system of the body has been partially clarified during the past 25 years by the demonstration in plasma of lipid-protein complexes that behave as soluble proteins(1,2,3). There is abundant evidence that body lipids are more active participants in metabolism than has been supposed(4) and that these lipoproteins play an active role in fat metabolism(5). It becomes of some importance to consider the mechanism of transport of these lipids across the body membranes.

Complexes of lipids and proteins were demonstrated in serous fluids 3 decades ago(6,7,9). Lipoprotein transport from plasma to lymph has been investigated in animals by several investigators(9,10,11,12,13) and both labeled albumin and cholesterol have been found in the aortic wall of the dog(14). The present study is an extension of this problem to measurement of the lipid and protein composition of serous fluids obtained from diseased patients with special reference to lipoproteins which have not been previously described in such fluids.

Materials and methods. Thirty-three fluids were collected from patients at the time of diagnostic or therapeutic taps. They were analyzed after a preliminary low speed centrifugation at 800 g for 30 minutes to remove fibrin and cells. The chemical methods were applied to a 1:20 Bloor's extract of the fluids. Total lipid was determined according to the method of Bragdon(15) and expressed as milligrams of dichromate reduced per 100 ml. Phospholipid was measured by the method of Youngburg, Fiske, and SubbaRow as described in Hawk, Oser, and Summerson(16). Total and free cholesterol were measured by the method of Sperry(17). Triglyceride was derived by correcting total dichromate reduced by cholesterol and phospholipid and introducing Bragdon's factor (17.7 mg of reduced dichromate equivalent to 1 mg of triglyceride). Nitrogen was determined by wet digestion and Nesslerization as described in Hawk, Oser, and Summerson (18). Albumin was separated by the method of Pillemer and Hutchinson(19). Low density lipoproteins were measured by the ultracentrifugation method of DeLalla and Gofman(20). The high density lipoproteins were separated by the method of Green, Lewis, and Page(21). For lipoprotein analyses aliquots of fluid were taken for preparatory centrifugation and the supernate was concentrated 8- or 10-fold. The second milliliter at the top of the preparative tube was routinely taken for a final density measurement in a one milliliter pycnometer constructed from a volumetric pipette. Migration rates were then corrected to densities 1.0630 and 1.2100 for the low and high density lipoproteins respectively. In practice, these corrections were small. The concentrations of lipoproteins were derived from the deflection areas on the analytical ultracentrifuge film. The analytical centrifuge was calibrated with the Spinco reference cell.

Results. The data are arranged in Table I according to source of fluids and clinical diagnosis. Those fluids which appeared to result from an increase in pressure gradient across the vascular membrane, either hydrostatic or osmotic, are separated from those fluids which appeared to result from an irritation to the capillary membrane, either inflammatory or neoplastic. This distinction is readily apparent in the peritoneal fluids, but it is uncertain in several of the pleural fluids. These indeterminant fluids are termed "mixed" because of the probable combination of pressure and irritative factors and are described in Table II along with a miscellaneous group of fluids from other serous cavities.

There were readily measurable quantities of both low and high density lipoproteins in

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TABLE I.

Type	Clinical diagnosis	Total protein	Albumin	Globulin	Total lipid‡	Total chol.	Lipid phos.	Triglyceride	Lipoproteins Low density	Lipoproteins High density
Peritoneal										
(A) ‘‘Pressure’’ n = 6*	Laennec's cirrhosis (4)† Mitral stenosis (2)	2,336 ± 894	1,381 ± 926	956 ± 520	2,217 ± 826	45.2 ± 19.1	1.79 ± .68	19.8 ± 18	65.3 ± 27.	66.5 ± 42.
(B) ‘‘Irritative’’ n = 5	Carcinomatosis (5)	4,016 ± 694	2,434 ± 761	1,909 ± 570	4,632 ± 1,680	83.6 ± 14.0	3.08 ± .29	80.6 ± 127	144 ± 16	60.8 ± 57.
(B)−(A)	p§	<.02	<.1	<.01	<.01	<.01	<.01	<.05	<.01	
Pleural										
(C) ‘‘Pressure’’ n = 4	Congestive heart failure (4)	906 ± 161	525 ± 215	381 ± 99	2,556 ± 1,069	9.6 ± 2.8	.88 ± .44	160 ± 32.4	13.2 ± 7.9	3.5 ± 4.2
(D) ‘‘Irritative’’ n = 5	Tuberculosis (2) Lymphosarc. (1) Pneumonia (2)	3,714 ± 1,541	1,849 ± 439	1,866 ± 1,252	3,914 ± 2,326	68.6 ±	2.40 ± 1.32	58.4 ± 84.4	136.4 ± 66.1	25.0 ± 20
(D)−(C)	p§	<.01	<.01	<.01	.05–.01	<.01	<.01	<.01‖	<.01	<.02

See text for method of classification. All results are expressed as mean milligrams % ± one stand. dev.

* n represents No. of fluids.

† No. of cases in each diagnosis is in parentheses.

‡ Expressed as mg dichromate reduced per 100 ml fluid.

§ Probability for observed value of ‘‘t.’’

‖ C > D.

all of the specimens except the edema fluid obtained by Southey tube. The high density lipoprotein determination could not be made on the pericardial fluid because of insufficient quantity. The ultracentrifugal patterns of the lipoproteins in these body fluids are similar to those of human plasma (Fig. 1). The low density material migrates principally as Sf 8-11, but it is often not homogenous (Fig. 1c).

The irritative fluids from both peritoneal and pleural cavities show significantly higher levels of low density lipoproteins, total cholesterol, and globulins ($p<0.01$) than do the pressure fluids (Table I). Concentrations of high density lipoproteins are similar. The mixed pleural fluids appear to have characteristics between those of the pressure and irritative groups (Table II). The peritoneal pressure fluids are more similar to the mixed group of pleural fluids than to the pressure group except for the low amount of triglyceride in the former. The 2 chylous fluids show large amount of triglyceride and low density lipoproteins migrating at rates of Sf 400 and faster.

Discussion. The large complexes demonstrated in these fluids were either derived from cellular debris or plasma lipoproteins. The differences in content between fluids may be related to differences in passage of molecules out of the body cavity rather than into the body cavity. The data of Oncley, Scatchard, and Brown(22) indicate that the minimum diameter of the high density lipoprotein molecule (mol. wt. 200,000) is 50 Å and that of the low density lipoprotein molecule (mol. wt. 1,200,000) is 185 Å. Although the pore theory of capillary permeability(22) limits the free diffusion of particles to those having diameters less than 20-45 Å, there is some evidence for the existence of a larger pore

TABLE II.

Type	Clinical diagnosis	Total protein	Albumin	Globulin	Total lipid*	Total chol.	Lipid phos.	Triglyceride	Low density	High density
Pleural ''mixed'' (see below) n = 5		1,742 ± 327	1,104 ± 212	638 ± 327	4,435 ± 1,720	32.2 ± 8.4	1.83 ± .33	140 ± 108	72.2 ± 20.8	27.2 ± 16.6
Joint fluids	Rheumatoid arthritis	3,902 4,575	2,794 2,300	1,112 2,275	 3,650	 91.5	4.92 2.32	 17	168 184	110. 59.0
Hydrocele fluids	Prostatic stricture Hernia	4,225 1,869	3,062 1,306	1,163 563	1,400 680	23.5 11.9	1.63 .92	88 0	12.5 0	31.0
Southey fluid	Diabetes mellitus Congestive heart failure	1,462	1,312	150	3,220	15.4	1.04	137	0	0
Pericardial	Laennec's cirrhosis (autopsy)	2,662					2.72		14.3	
Chylous pleural fluids	Lymphoma	3,993 2,869	3,150 1,893	843 776	17,600 17,450	110. 64.7	4.00 4.88	750 785	98 34	S_f 35-100: 45.1 S_f 100-400:133.5 S_f 35-100: 45.0 S_f 100-400:101.0

See Table I and text for explanation. ''Mixed'' fluids appeared to have both ''pressure'' and ''irritative'' elements in their pathogenesis, *e.g.* congestive heart failure complicated by pulmonary infarction, neoplasia or pneumonia.

* Expressed as mg dichromate reduced per 100 ml fluid.

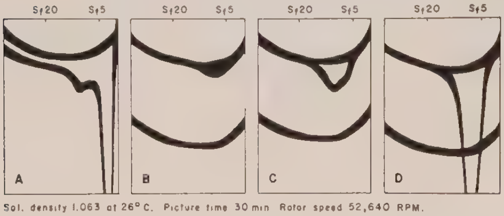

FIG. 1. Comparison of high density ultracentrifugation patterns. A represents a serum pattern from a 51-year-old man who had experienced a myocardial infarction. The serum was concentrated 4 times. B, C, and D represent patterns from pleural "pressure," "mixed," and "irritative" groups respectively, concentrated 8 times. The lower patterns in frames B, C, and D represent the second one-half milliliter from the preparative centrifugation tube. These samples were run simultaneously in a wedge window cell. The base lines are superimposed.

(24). It is also possible that the pathological process enlarges the diameter of the pores. There are data for such a phenomenon in dogs(13). Electronmicroscopic observations (25) suggest that lipoproteins pass through intima by a mechanism similar to phagocytosis.

The peritoneal fluids have higher solute concentrations than the pleural fluids of the same presumptive pathogenesis. This is consistent with studies of infused dextran in dogs (24) and may reflect the fact that peritoneal pressure fluid traverses principally the liver capsule and not the portal capillary bed(26). Despite the similar total lipid concentrations in peritoneal and pleural pressure fluids, concentration of triglycerides was low in the former whereas concentration of cholesterol and lipid phosphorous was low in the latter. The high amount of triglyceride in the pleural pressure fluids was not manifest as free floating lipid.

The miscellaneous joint, hydrocele, pericardial, and edema fluids show small amounts of lipoproteins resembling those of plasma. The absence of demonstrable lipoprotein in the edema fluid obtained by Southey tube despite a significant protein content sheds no light on the debate concerning the existence of lipoprotein in normal extravascular fluid (10,27,28).

The chylous fluids described in Table II must be considered separately since they were each a consequence of lymphoma which may have caused a rupture or a congestion of the thoracic duct leading to a mechanical transfer of thoracic duct lymph into the pleural space. The composition of these chylous fluids is similar to that of cannulated thoracic duct lymph as in fat fed dogs(9) and that found by Peters and Man(29). The low density components of such fluids resemble those of post-prandial serum and the lipid content is comparable.

Summary. Lipids and proteins exist in peritoneal, pleural, joint, hydrocele, and pericardial effusions in large complexes having migrational characteristics in the ultracentrifuge similar to plasma lipoproteins. Concentration ranges from 5 to 50% of that in plasma. Classification of fluids according to traditional concepts of pressure and irritation reveals that irritative fluids tend to have higher concentrations of low density lipoproteins, total cholesterol, and globulin than do pressure fluids. The origin of these large lipoprotein aggregates is uncertain. The various possibilities are discussed.

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1. Macheboeuf, M. A., *Bull. Soc. Chim.*, 1929, v45, 662.

2. Pedersen, K. O., *Upsala,* 1945, v33.

3. Gofman, J. W., Lindgren, F. T., Elliott, H., *J. Biol. Chem.*, 1949, v179, 973.

4. Jones, H. B., Gofman, J. W., Lindgren, F. T., Lyon, T. P., Graham, S. M., Strisomer, B., Nichols, A. V., *Am. J. Med.*, 1951, v11, 358.

5. Man, E. B., Albrink, M. J., *Yale J. Biol. and Med.*, 1956, v29, 316.

6. Man, E. B., Peters, J. P., *J. Clin. Invest.*, 1933, v12, 1031.

7. Foord, A. B., Youngberg, G. E., Wetmore, V., *J. Lab. and Clin. Med.*, 1928-29, v14, 417.

8. Bruger, M., *Am. J. Clin. Path.*, 1935, v5, 384.

9. Page, I. H., Lewis, L. A., Plahl, G., *Circ. Res.*, 1953, v1, 87.

10. Kellner, A., Nat. Acad. Sci., *Nat. Res. Council Publ.*, Symp. on Atherosclerosis, 1955, v42, 338.

11. Courtice, F. S., Morris, B., *J. Exp. Phys.*, 1955, v40, 138.

12. Morris, B., Courtice, F. S., *ibid.*, 1955, v40, 149.

13. Shirley, H. H., Jr., Wolfram, C. G., Wasserman, K., Mayerson, H. S., *Am. J. Phys.*, 1957, v190, 189.

14. Duncan, L. E., Buck, K., *Circ. Res.*, 1959, v7, 765.

15. Bragdon, J. H., *J. Biol. Chem.*, 1951, v190, 513.

16. Hawk, P. B., Oser, B. L., Summerson, W. H., *Prac. Physiol. Chem.*, 13th Ed., 1954, 54 and 581.

17. Sperry, W. M., Webb, M. A., *J. Biol. Chem.*, 1950, v187, 97.

18. ———, *ibid.*, 548, 593.

19. Pillemer, L., Hutchinson, M. C., *ibid.*, 1945, v158, 299.

20. DeLalla, O. F., Gofman, J. W., *Meth. Biochem. Anal.*, 1954, v1, 549.

21. Green, A. A., Lewis, L. A., Page, I. H., *Fed. Proc.*, 1951, v10, 191.

22. Oncley, J. L., Scatchard, H., Brown, A., *J. Phys. and Colloid Chem.*, 1947, v51, 184.

23. Pappenheimer, J. R., *Phys. Rev.*, 1955, v33, 387.

24. Grotte, G., *Acta Chirurg. Scand.* Suppl. No. 211, 1956.

25. Buck, R. C., *Circulation,* 1957, v16, 484.

26. Volweiler, W., Bollman, S. L., Grindlay, J. H., *Proc. Staff Meeting, Mayo Clinic,* 1950, v25, 31.

27. Marble, A., Field, M. E., Drinker, C. K., Smith, R. M., *Am. J. Physiol.*, 1934, v109, 467.

28. Geyer, G., Keible, E., Kolbl, H., *Exp. Med.*, 1953, v122, 1.

29. Peters, J. P., Man, E. B., *Metabolism,* 1953, v2, 30.

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Induction of Regeneration of Body Parts in the Lizard, Anolis.*† (26549)

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The non-regenerating forelimb of the adult frog (Rana) can be caused to regrow after amputation if the fresh wound is repeatedly irritated by harmful agents(1,2). Regeneration can also be evoked if the number of nerve fibers at the wound surface is increased by deviating additional nerves from other regions into the forelimb(3); and, indeed, the method of traumatism does not call forth a growth if the stump is denervated(4). The theory behind my experiments emerged from previous studies on the important controlling influence of the nerve on regeneration of body parts in the newt and other lower vertebrates (5). The extremities of the newt regenerate after amputation provided the number of nerve fibers at the amputation surface satisfies certain threshold requirements; quantities of fibers below the threshold value are ineffective. The threshold studies raised the possibility that the loss of power to regenerate which the frog's limb suffers at metamorphosis may be due, among other causes, to quantitative inadequacy of the available nerve supply, a theory substantiated by the nerve augmentation experiments.

Vertebrates higher than the frog also do not regenerate their limbs. The present study reports experiments begun in 1957 to evoke regrowth in a representative of a higher vertebrate group, the reptile, *Anolis carolinensis*, by increasing the nerve supply at a wound surface. The lizard does not regrow§ limbs but does regenerate the tail provided the spinal cord is present at the amputation surface. The experimental design was based on the following questions: Do the limb nerves of the lizard also have the quality to support regeneration given appropriate circumstances, and, are the tissues of the limb stump able to respond to the nerve?

Materials and methods. To answer these questions 2 experiments were devised. In one case, hindlimb nerves were deviated into a surface wound area of the tail, namely, into an organ field capable of regrowth (Fig. 1); in the other, nerves of one hindlimb were deviated to the opposite amputated limb to increase the fiber quantity at the wound surface (Fig. 2). In each instance the sciatic nerve and its major branches were dissected to the upper thigh region and then deviated according to procedures adopted in my previous experiments with the frog(3).

Nerve deviation to the tail. In the case of deviations to the tail both sciatic nerves and their branches were dissected to the knee and then redirected under the skin to a dorsal region of the base of the tail from which approximately 5 mm^2 of skin was removed and the underlying tendons and muscles cut repeatedly to increase the severity of the injury (Fig. 1). In 18 animals, 8 showed growths beginning about 6 weeks after operation. The growths varied in shape and size; the largest was approximately 6.6 mm (Fig. 3) and the smallest about 1 mm. Initial histological preparations showed abundant growing nerve fibers, connective tissue and some regenerating muscle near the base of growth. In some of the negative cases a small pad of tissue developed under the regenerated skin. In 5 instances in which a wound was made but no nerves deviated ordinary wound healing ensued without any sign of enlargement.

Nerve deviation to the opposite limb. In the second experimental series, the left sciatic nerve and its branches to a level below the

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† The article of Simpson in this issue of the Proceedings, submitted for publication at the same time, reports induction of regeneration in a lizard by deviation of spinal cord to the hindlimb.

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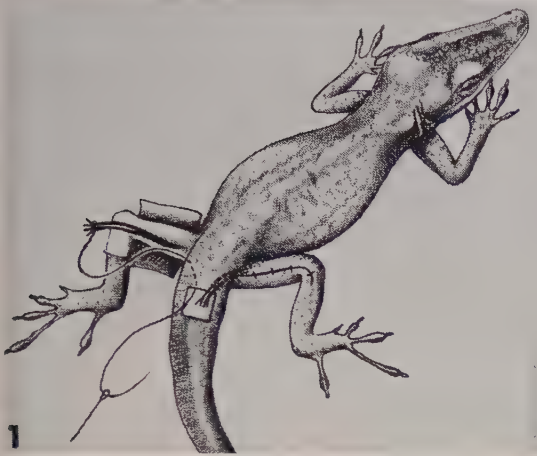
§ Kudokotsev(9) recently observed regenerative responses in the hindlimb of the desert skink which could be enhanced by enlarging the surface of amputation.



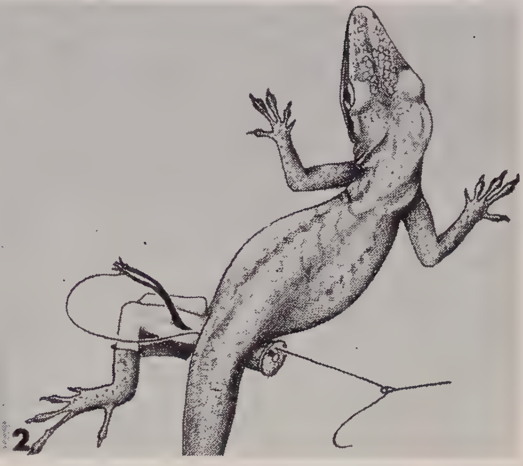


FIG. 1. Deviation of sciatic nerves to wound in tail. Right sciatic in place, left in process of deviation. × 0.8.

FIG. 2. Deviation of left sciatic nerve to right amputation stump. × 0.8.

knee were redirected under the spinal column and thence along the path of the opposite sciatic nerve to the freshly amputated stump (about 4 mm from base) of the right hindlimb (Fig. 2). In 9 of 21 cases, regeneration occurred from the amputation surface. The growth was initiated about 6 to 12 weeks after nerve deviation. The first signs of growth (Fig. 4) were swelling underneath a deeply pigmented wound epithelium and increased sensitivity of the wound skin to mechanical stimulation. Then growth was rapid for approximately 8 weeks. The largest growth achieved was approximately 4 mm and in this instance the distal end also became flattened suggesting a foot rudiment (Fig. 4). In general, the growths were smaller and less well-developed than those seen in the frog. In 9 controls involving either simple amputation or deviation of a resected piece of sciatic nerve there was no growth(6). The wound skin assumed a light grayish cast from the start and the end of the stump became atrophic.

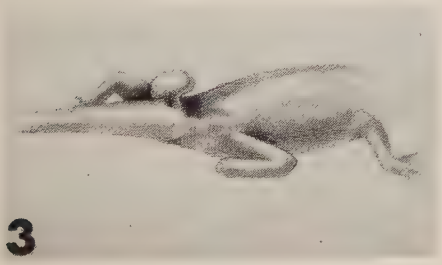


FIG. 3. Growth in tail evoked by sciatic nerve deviations (see Fig. 1). Anolis 16. × 1.

Conclusions. The results show that the nerves of the lizard do have the necessary quality to evoke regeneration of a body part. Secondly, they show that the limb is capable of regeneration provided an adequate neural stimulus is available. The fact that fewer positive instances of limb regeneration were obtained than in the frog and that the growths

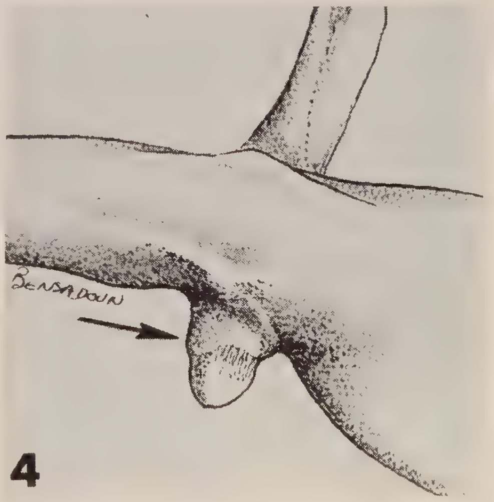


FIG. 4. Regeneration of right hindlimb after deviation of left sciatic to amputation surface (see Fig. 2). Arrow points to orignal amputation surface. Anolis 56. × 2.3.

were more limited and heteromorphic intimates that the lizard limb is more refractory to the nerve stimulus. The results bear out the expectation previously drawn(3,7,8) that adequate "dispositions" may be found to cause regeneration in forms higher than the frog and raise the hope that even the limb of mammals may some day be caused to regrow.

Summary. The amputated hindlimb of the lizard, *Anolis carolinensis,* incapable of regenerating, may be caused to grow by augmenting the nervous supply of the wound surface with additional nerves deviated from the opposite hindlimb. In addition, deviation of hindlimb nerves into the tail region also induced growth responses. The conclusion is drawn that the reptilian nerve does have the growth-evoking quality exhibited so well by nerves in lower forms and that reptilian body parts do have the power to regrow provided, among other conditions, that the nerve stimulus is quantitatively adequate.

I am grateful to Paula di Santo Bensadoun for drawings in Figs. 1, 2, and 4 and to E. Bailey for Fig. 3.

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1. Polejaiev, L. W., *Biol. Rev.,* (Cambridge Philos. Soc.), 1946, v21, 141.
2. Rose, S. M., *J. Morph.,* 1945, v77, 119.
3. Singer, M., *J. Exp. Zool.,* 1954, v126, 419.
4. Singer, M., Kamrin, P. K., Ashbaugh, A., *ibid.,* 1957, v136, 35.
5. Singer, M., *Quart. Rev. Biol.,* 1952, v27, 169.
6. Barber, L. W., *Anat. Rec.,* 1944, v89, 441.
7. Spallanzani, A., *An essay on animal reproduction.* London, 1769.
8. Morgan, T. H., *The Harvey Lectures* (Lippincott, Philadelphia), 1905-06, 219.
9. Kudokotsev, V. P., *Akad. Nauk SSSR, Doklady,* 1959, v126, 1141.

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Induction of Limb Regeneration in the Lizard, *Lygosoma laterale*, by Augmentation of the Nerve Supply.*† (26550)

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Regeneration of appendages in urodele amphibians is dependent on presence of an adequate nerve supply(1). The same holds true for anuran larvae(2). Singer(3) showed that the influence of the nerves in Triturus limb regeneration was of a quantitative rather than qualitative nature. That is, the number of nerve fibers per unit area of tissue determines whether or not the limb will regenerate. He suggested that the absence of limb regeneration in the higher vertebrates might be due to lack of a sufficient nerve complement. To test this hypothesis, Singer(4,5) augmented the nerve supply of the normally non-regenerating forelimb of the postmetamorphic frog and obtained regeneration.

The adult lizard presents a unique case, in that the limbs do not regenerate, yet the tail regenerates freely. This apparent paradox can be most readily explained by the fact that the tail, which contains the spinal cord, obviously has a larger nerve supply than the limb. If this assumption is valid, one should be able to induce regeneration of the limb by augmenting its nerve supply.

The present study emphasizes the importance of nerves in lizard regeneration. The nerve supply of the rear limb of the lizard was augmented by deviating the spinal cord from the tail into the limb. A limited amount of true limb regeneration was obtained.

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† A more detailed account of the histological aspects of this study will be published later. The results were first presented as a progress report at Fourth Developmental Biology Conference, Alligator Point, Fla.

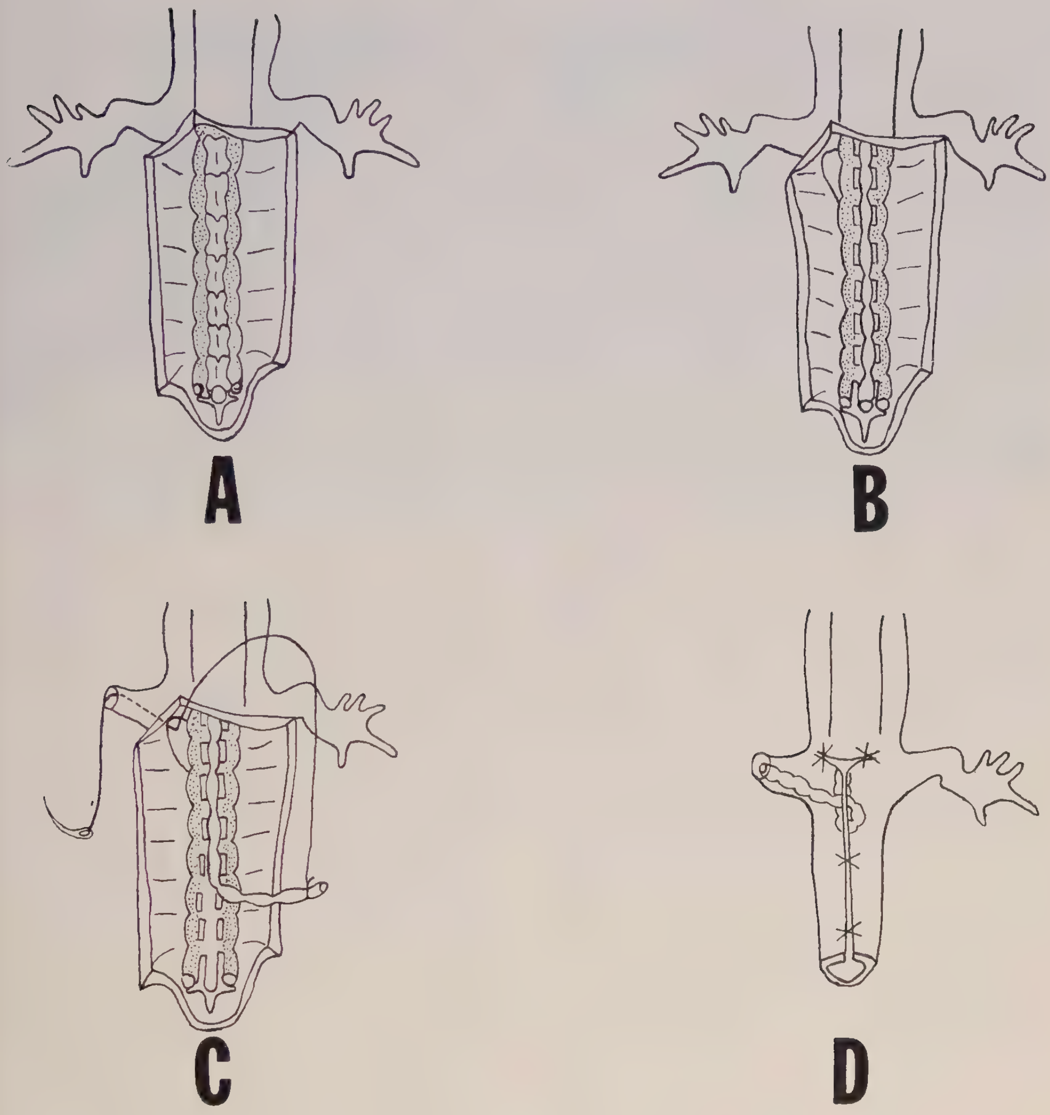

FIG. 1. Diagram of operation.

Materials and methods. In these experiments, adult brown skinks (*Lygosoma laterale*) were maintained in individual glass stacking dishes at room temperature (70-75°F) under controlled light conditions, and were fed a diet of meal worms, *Tenebrio* sp.

The experimental series consisted of 16 lizards in which the spinal cord was surgically deviated into the left rear limb. The major steps in the operation are diagrammed in Fig. 1. In each case, the lizard was first curarized with an intraperitoneal injection of tubocurarine chloride in saline. The tail was amputated approximately 2 inches from its base and a mid-dorsal incision was made in the integument. Working along this incision the vertebral column was exposed (Fig. 1 A). The roofs of the vertebrae were removed, exposing the spinal cord (Fig. 1 B). The limb was then amputated half-way between the body wall and the distal end of the femur. A suture was passed through a channel leading into the limb. The free end of the suture was tied to the posterior end of the spinal

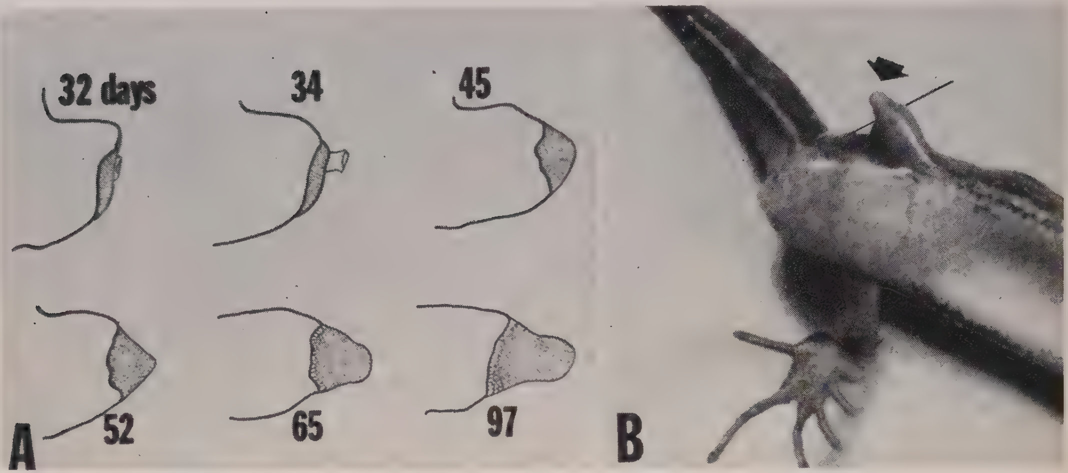

FIG. 2. Experimental lizard E-1. (A) Camera lucida drawings showing successive stages in development of the regenerate. × 7. (B) Photograph of regenerate (arrow) 106 days following operation. Broken line indicates original level of amputation. × 2.

cord (Fig. 1 C) and the cord pulled into the limb. The dorsal incision in the tail was then closed with sutures (Fig. 1 D).

Two control groups were set up. In both groups the control limbs were amputated at the same level as were the experimentals. In the first group (basic controls), simple amputation of the left rear limb was performed in 21 lizards. This series was established to stage the histology of healing in the normally non-regenerating limb. The second group (experimental controls) consisted of 17 lizards which underwent the same operation as did the experimentals, with the one exception that the spinal cord was not deviated into the limb. Tail muscle was transplanted to some of the limbs in this series. This was done to determine whether or not tail tissue, other than spinal cord, might possess inductive properties.

Results. None of the limbs in the 2 control series displayed a regenerative response. Histological examination of normal healing in the non-regenerating control limbs revealed considerable *de*differentiation of the limb tissues, with the exception of bone. However, these limbs were characterized by a premature *re*differentiation of the dedifferentiated cells into scar tissue. A cartilage callus also formed around the cut end of the unaltered bone.

Eight of the 16 limbs into which the spinal cord had been deviated, showed regenerative responses. Two of these limbs resulted in positive cases of limited limb regeneration. One of the regenerates is shown in Fig. 2. The first external indications of regeneration appeared 34 days following operation. The blastema was very soft and had assumed the shape of a small cone by 52 days. By 65 days, the blastema had elongated and was still soft and flexible. The regenerate had reached its maximum length (1.1 mm) by 97 days. At 106 days (Fig. 2 B) the animal was sacrificed and the regenerate removed for histological examination which revealed that bone and muscle had undergone extensive breakdown, and the regenerate fulfilled the requirements of a limb regenerate. Regenerated muscle fibers were present in the regenerate and were grouped in a manner normal for limb muscle. A regenerated cartilagenous model of the femur could be seen extending from the area of bone breakdown. The cartilage model had formed *de novo,* rather than by appositional layering from the periosteum. This was evidenced by the spatial arrangement of the chondrocytes and the presence of a well formed epiphyseal plate. The model was also undergoing periosteal ossification at its base.

The other 6 experimental limbs showed regenerative responses, but did not proceed as far as the one just described. Study of Bodian stained sections of the experimental series showed that the best regenerate was associated with a definite increase in nerve fiber content.

Discussion. Previous workers have succeeded in evoking growth responses in lizard limbs(6,7). In each case, however, the resulting growths proved to be tail-like in nature.

In the present study deviation of the spinal cord (a source of additional nerves) into the limb of *Lygosoma* induced a limited amount of true limb regeneration. Therefore, this study demonstrates that the limb tissues *per se* are still capable of responding to the inductive influences of nerves. It also emphasizes the validity of the neurological theory by extending it to the next higher vertebrate group, the Reptilia.

The results can be interpreted in at least two ways. 1) Possibly the nerves of the normal limb have lost their ability to induce regeneration, while the nerves of the spinal cord have retained this property. 2) Possibly the limb nerves have the ability to induce regeneration, but fibers are not present in sufficient numbers. Since a considerable amount of dedifferentiation occurred in the control limbs, it would seem that the limb nerves may still have the ability to take part in regeneration. If the latter is true, deviation of the spinal cord into the limb merely increased the nerve complement above the threshold that allowed regeneration to occur. Therefore, the results favor the second interpretation. Results of previous work on limb regeneration in Triturus(3) and induced limb regeneration in the postmetamorphic frogs(5) also favor this interpretation. This explanation is even more acceptable in light of recent findings of Singer(8).

Summary. The spinal cord was deviated from the tail into the rear limb of the lizard, *Lygosoma*. In those cases where the deviated cord retained its integrity and augmented the nerve complement of the limb, a limited regenerative response occurred. Therefore, the tissues of the limb are still capable of responding to the inductive influences of nerves. Histological examination of the regenerates revealed that they fulfilled the requirements of true limb regeneration. Experimental control limbs which underwent the same treatment with the exception that the cord was not deviated into them, failed to show a regenerative response. This study strongly suggests that lack of an adequate supply of nerve tissue imposes one, if not the major, restriction on limb regeneration in this lizard.

The author wishes to express his appreciation to Dr. Merle Mizell for encouragement and guidance during this study.

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1. Singer, M., *Quart. Rev. Biol.,* 1952, v27, 169.
2. Schotté, O. E., Harland, M., *J. Exp. Zool.,* 1943, v93, 453.
3. Singer, M., *ibid.,* 1946, v101, 299.
4. Singer, M., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 413.
5. Singer, M., *J. Exp. Zool.,* 1954, v126, 419.
6. Guyénot, E., Matthey, R., *Arch. F. Entw. Mechan.,* 1928, v113, 520.
7. Rose, S. M., *Year Book Am. Philos. Soc.,* 1951, 134.
8. Singer, M., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 106.

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Immunoelectrophoresis of Serum in Progressive Systemic Sclerosis (Diffuse Scleroderma).* (26551)

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Since immunoelectrophoresis was introduced by Grabar and Williams(1) about 30 distinct proteins have been recognized in normal human serum(2), and as Grabar predicts many more will be described(3). These proteins appear in immunoelectrophoresis as specific precipitation arcs which can be identified by their characteristic mobility or configuration, by special staining procedures, or by absorbing the immune antiserum with certain purified proteins. The present study is concerned with paper strip and immunoelectrophoresis of the serum of patients with progressive systemic sclerosis (P.S.S.), in order to further characterise the protein abnormalities in this disorder(4,5).

Materials and methods. The serum samples used in this study were obtained from 15 healthy individuals and 15 patients with well-documented progressive systemic sclerosis in whom the diagnosis of scleroderma was confirmed by skin biopsy. Serum was separated from venous blood and kept frozen from several days to 2 months prior to examination. Immunoelectrophoresis was performed with Scheidegger's micro apparatus, using 25 × 75 mm microscopic slides(6). A 2% agar solution prepared in sodium barbital buffer, pH 8.2, ionic strength 0.02, was used as a medium for electrophoretic separation. Two parallel wells were cut out in the agar plate; one was filled with the pathological serum and the other with a normal serum. After 35 minutes of electrophoretic separation under a potential of 6 V/cm, a central trough was cut out and the immune antiserum was introduced. Diffusion was allowed to take place for 24 hours and the slides then washed in buffered saline for 24-48 hours. After drying, the slides were stained with azocarmin G.

All the immunoelectrophoretic studies were performed with antisera #511 and #512 from horses immunized with whole normal human serum, obtained from the Pasteur Institute in Paris. On several occasions, however, rabbit anti-alpha$_2$ macroglobulin serum and rabbit anti-normal human serum, obtained from Behringwerke in Marburg, were also used.[‡] The results presented in Table I are those obtained using undiluted sera and antisera. An attempt to obtain clearer patterns by diluting either the serum or antiserum usually failed to improve the sharpness of the arcs.

Paper electrophoresis was performed with a Spinco Model R paper electrophoresis apparatus, and evaluation of the stained paper strips stained by alcoholic bromphenol blue was done by a Spinco Analytrol instrument. Total serum protein was determined by a modified biuret method(7). The results are given in Table I.

Results. The different immunoelectrophoretic precipitation lines obtained with antiserum #511 are illustrated in Fig. 1. The upper pattern is of a patient with progressive systemic sclerosis and the lower is of a normal control.

The following precipitation lines were found both in the normal controls and in the patients under study. *Albumin*: Two prealbumins, rho$_1$ and rho$_2$, were observed together only once. In most cases they were either absent or only one of them was present. (They were absent in the sera illustrated in Fig. 1.) The serum albumin formed a prominent arc which extended to the region of the alpha$_2$ globulin. *Alpha globulin*: The alpha$_0$ globulin appeared as a hazy complex inside the curvature of the albumin arc. This com-

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plex has been referred to as the × fraction and is identical with orosomucoid(3). The alpha$_1$ globulin appeared as a large, broad line which was accentuated at its cathodic extremity. According to Burtin(3), this line does not show important variation even in pathological sera. Our findings concurred. In the alpha$_2$ region, 3 components were found. The arc nearest to the antibody trough was identified with haptoglobin, while the outer arc showed a slower mobility and was identified as alpha$_2$ macroglobulin. Between these precipitation lines, a third line was sometimes present. This is probably the ceruloplasmin. Because this line was not always present even in the controls, it does not figure in Table I. *Beta globulin*: With antiserum #511, several beta$_1$ lines were found. Of these we shall mention the beta$_1$ siderophilin, an unidentified beta$_1$ globulin, and the beta$_1$ lipoprotein complex which originates from the antigen well and is the farthest from the antibody trough. This latter gives a positive staining with Sudan black. In the beta$_2$ region, the beta$_2$M and the beta$_2$A globulins run parallel to the gamma globulin. The beta$_2$A was sometimes missing in the controls as well as in the pathologic sera, and therefore does not figure in the Table. *Gamma globulin*: The gamma globulin fraction usually presented a long, asymmetric single line which extended up to the alpha$_2$ region. In 4 normal sera, however, there was a splitting at the anodic extremity.

The results of immunoelectrophoretic examination of the sera from 15 patients with progressive systemic sclerosis, as compared to normal controls, are given in Table I. Pertinent changes were observed in alpha$_0$, alpha$_2$ and gamma fractions. The alpha$_0$ was increased in 7 out of 15 cases. This manifested itself by a thick and clear precipitation line. The alpha$_2$ macroglobulin was increased

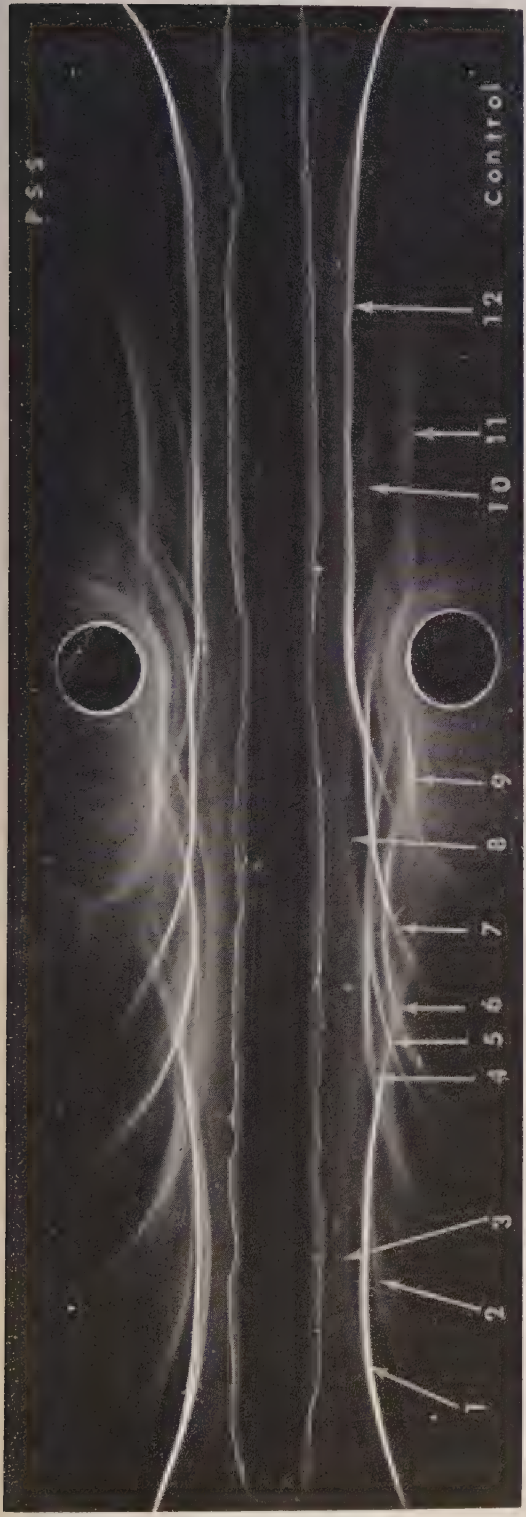

FIG. 1. Immunoelectrophoretic patterns of a patient (No. 6) with progressive systemic sclerosis (PSS) and a normal control. 1. Albumin. 2. Alpha$_0$ globulin. 3. Alpha$_1$ globulin. 4. Alpha$_2$ haptoglobin. 5. Ceruloplasmin (2). 6. Alpha$_2$ macroglobulin. 7. Siderophilin. 8. Beta$_1$ globulin. 9. Beta$_1$ lipoprotein. 10. Beta$_2$A globulin. 11. Beta$_2$M globulin. 12. Gamma globulin. Note splitting of the gamma globulin in the pathological serum.

TABLE I. Immuno- and Paper Strip Electrophoresis of Serum Proteins in Progressive Systemic Sclerosis.

		Immuno-electrophoresis						Paper strip electrophoresis				
Patient	Age, race, sex	α_0	α_2 haptogl.	α_2 macrogl.	β_2M globulin	γ globulin	Total protein	Albumin†	α-1	α-2	β	γ
1	37W ♀	+	+	+	↑	>—	6.63	3.96	.27	.51	.71	1.18
2	42W ♂	+	↑	↑	+	+	6.95	3.68	.36	.76	.93	1.21
3	52W ♀	↑	+	↑	+	>—	7.54	3.79	.32	.79	.83	1.82
4	38W ♀	+	+	↑	+	>—	7.81	4.74	.40	.69	.75	1.23
5	70W ♀	↑	↑	↑	+	+	6.96	3.77	.23	.60	.97	1.40
6	26W ♀	↑	+	↑	↑	>—	6.27	3.33	.28	.84	.77	1.05
7	15W ♀	+	+	↑	+	+	6.83	3.80	.29	.76	.70	1.27
8	37W ♀	↑	↑	↑	↑	+	7.05	4.59	.10	.34	.63	1.40
9	35W ♀	↑	+	+	↑	>—	8.83	3.58	.35	.50	.86	3.54
10	44W ♀	+	+	↑	↑	>—	6.58	3.47	.25	.72	.76	1.38
11	68W ♂	+	+	+	+	+	6.46	3.07	.29	.87	.65	1.58
12	58W ♂	↑	+	+	+	+	7.86	4.36	.31	.68	1.05	1.48
13	36W ♂	+	+	+	↑	>—	6.81	2.83	.39	.76	.95	1.87
14*	35W ♀	↑	↑	↑	+	+	7.52	3.44	.29	.54	.80	2.46
15	47W ♀	+	+	+	+	>—	7.85	3.52	.27	.99	1.06	2.01
					Normal range		6.15–8.11	3.81–5.61	.19–.35	.31–.75	.42–.90	.52–1.40
					S.D.		.49	.45	.04	.11	.12	.22

* Sjögren's syndrome and P.S.S.

† All values in g %.

+, Normal length, thickness, and configuration of precipitin arc.

↑, Increase in thickness and sometimes length of precipitin arc.

>—, Splitting of precipitin arc.

in 9 cases; beta$_2$M globulin was increased in only 6 cases. Quantitative changes could not be observed in the gamma globulin fraction; however, a splitting of the anodic extremity of the precipitation line was a notable finding in 8 cases. No deviations from the normal were noted in serum albumin, alpha$_1$ globulin, beta$_1$ siderophilin, and beta$_1$ globulin.

Table I also provides the results of paper strip electrophoretic examination of the sera from these patients. There was a slight increase in alpha$_1$ globulins in 2 patients and in the alpha$_2$ globulins in 4. A significant increase in gamma globulins was observed in 8 patients, ranging from 1.48 g % to 3.45 g %. No strict relationship could be found between the variation in the composition of the serum proteins revealed by immunoelectrophoresis and paper strip electrophoresis.

Discussion. Our results indicate that immunoelectrophoresis complements paper strip electrophoresis in revealing delicate variations not detected by the latter. Analysis of the different precipitation lines is not always easy, especially where quantitative aspects are concerned. According to Burtin(3), an increase of a certain protein fraction in a complex protein mixture, such as serum, manifests itself in the immuno-electrophoretic diagram by an increase in thickness or length or clearness of the particular precipitation line. Sometimes this line may split in its center into several components which remain united at their extremities. Being aware of the limits of this method of quantitation, we listed a protein as increased only when the corresponding precipitation line was markedly thicker and sometimes also longer than the corresponding line of the control serum.

We could thus observe an increase in the alpha$_0$ globulin and in the alpha$_2$ macroglobulin fractions which was not evident in paper strip electrophoresis. The increase in the alpha$_0$ fraction, probably identical with the seromucoid or orosomucoid, is of particular interest, because the orosomucoids have been reported as being elevated in the active stage of other systemic rheumatic disorders(8). The importance of the increase in the alpha$_2$ macroglobulin is not clear. This line has been found to be increased also in rheumatoid arthritis(9).

The splitting of the gamma globulin arc at its anodic extremity is also of particular interest. Edelman *et al.*(10) in an extensive immunological study of human gamma globulin reported that different purified human gamma globulin fractions give 2 precipitation lines with sera of rabbits immunized against certain pathological human sera. Similar results were reported by Burtin with special horse anti-human sera(3). This indicates that normal human gamma globulin has at least 2 antigenic determinants. The more common splitting of the gamma globulin in progressive systemic sclerosis, as compared to the control group, suggests that the morbid process may lead to an increase in the lesser of these antigenic determinants. Similar splitting of the anodic extremity of the gamma globulin has been described in hepatic cirrhosis(11).

Immunoelectrophoretic studies of sera from 8 patients with rheumatoid arthritis were reported by Cleve and Hartmann(12) who found an increase in the alpha$_1$ globulin, alpha$_2$ haptoglobulin, and in the beta$_2$M fraction. Similar observations were reported by Seligman(3) in systemic lupus erythematosus. Here however, the outstanding feature was a diminution or complete absence of the beta$_1$A fraction. The findings in P.S.S. differ from those in the above cited studies in that a significant increase in alpha$_2$ macroglobulin fraction was found. This and the less frequent increase in the beta$_2$M fraction may be related to the use of different antisera.

Immunoelectrophoresis adds some interesting data to the variations in composition of the serum proteins in progressive systemic sclerosis. It does not, however, give a particular picture characteristic of this disease to warrant its routine use for diagnostic purposes. The increase in the alpha$_2$ macroglobulin in this disease and in rheumatoid arthritis may point to a relationship worthy of further investigation.

Summary. Immuno- and paper strip electrophoresis were performed on the sera of 15 healthy individuals and 15 patients with progressive systemic sclerosis. An increase was observed in the alpha$_1$ globulin in 7 patients, in the alpha$_2$M globulin in 9, and in beta$_2$M globulin in 6. Splitting of the anodic extremity of the gamma globulin was more common in the pathological sera than in the controls. No relationship was found between these changes and those observed with paper strip electrophoresis.

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1. Grabar, P., Williams, C. A., *Biochem. Biophys. Acta,* 1953, v10, 193.

2. Heremans, J. F., *Clin. Chim. Acta,* 1959, v4, 639.

3. Grabar, P., Burtin, P., *Analyse Immuno-électrophorétique.* Paris: Masson et Cie, 1960.

4. Ogryzlo, M. A., Maclachlan, M., Dauphinee, J. A., Fletcher, A. A., *Am. J. Med.,* 1959, v27, 596.

5. Rodnan, G. P., Etiology and pathogenesis of progressive systemic sclerosis and dermatomyositis. In *Hahnemann Symposium on Inflammation and Diseases of Connective Tissue,* to be published by W. B. Saunders Co., Philadelphia. in press.

6. Scheidegger, J. J., *Internat. Arch. Allergy,* 1955, v7, 103.

7. Gornall, A. G., Bardawill, C. J., David, M. M., *J. Biol. Chem.,* 1949, v177, 751.

8. Decker, B., McGuckin, W. F., McKenzie, B. F., Slocumb, C. H., *Arth. and Rheumat.,* 1960, v3, 49.

9. Schultze, H. E., Personal Communication.

10. Edelman, G. M., Heremans, J. F., Heremans, M. T., Kunkel, H. G., *J. Exp. Med.,* 1960, v112, 203.

11. Scheidegger, J. J., Zahnd, G., *Helvet. Med. Acta,* 1957, v24, 499.

12. Cleve, H., Hartmann, F., *Klin. Wschr.,* 1957, v35, 334.

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Aspermatogenesis Induced by Testicular Antigen Uncombined with Adjuvant. (26552)

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Immunologically induced aspermatogenesis in the guinea pig follows intracutaneous injection of testicular antigen combined with complete adjuvant of the Freund type(1,2,3). Previous investigations have indicated that one or a limited number of injections of testicular homogenate or of sperm alone fails to induce germinal lesions(1,2) as does administration of antigen and adjuvant into separate sites on different sides of the animal (1). Considerable emphasis has thus been attributed to the role of adjuvant in the reaction(4,5,6), and at least one theory has been proposed which requires previous combination and injection of antigen and adjuvant together into the same sites(7, *cf.* 8,9).

Evidence from other systems involving delayed-type hypersensitivity, however, suggests that incorporation of antigen into paraffin oil adjuvants may not be essential for ultimate elicitation of specific germinal responses. The early demonstration, for example, of induced disseminated encephalomyelitis in monkeys was accomplished by many injections of brain antigen without adjuvant (10). Stone and Freund (11) have recently shown that the delayed-type skin-hemorrhage sensitivity of guinea pigs, developed in response to separate injections of ovalbumin and complete adjuvant, is different depending on whether injections are made on the same or on different sides of the nuchal mid-line. Similar analyses have been applied to the aspermatogenic syndrome; the present report concerns results of 2 types of experimental procedure: (a) frequent injections of minute amounts of testicular homogenate and (b) administration of antigen and adjuvant in separate sites on the same dorsal quadrant of the guinea pig. A preliminary note concerning the latter study has been published(12).

Material and methods. Adult male guinea pigs of Hartley and mixed outbred strains were intracutaneously injected in the nuchal region as scheduled in Tables I and II. Homologous testicular homogenate in saline (1:1) was prepared from normal adult gonads as previously described(2). Adjuvant was of the Freund type which includes Bayol F paraffin oil, Arlacel A emulsifying agent, and mycobacteria (Difco Laboratories, Detroit, Mich.).

In the first, low-dose series, testicular homogenate was repeatedly injected in amounts and at rates shown in Table I. At sacrifice, 21 to 157 days after first injection, body weights indicated normal growth. Testicular tissue was fixed in formol-Zenker, sectioned at 5 μ, and stained in haematoxylin-eosin or Himes-Moriber trichrome stain(13). Three animals were unilaterally castrated at day 63 and sacrificed one month later. Scoring of germinal lesions follows the procedure previously adopted(2) and indicates increasing severity from 1 to 4.

Each guinea pig in the second, separate-site series received 0.5 ml testicular homogenate in saline and 0.5 ml complete adjuvant in locations separated by distances 2.0 cm or greater (Table II). In some animals, antigen was injected adjacent to the dorsal midline while adjuvant was administered laterally; in others, the positions were reversed. In 4 controls (Nos. 20-23), antigen was injected on the right side and adjuvant on the left of the mid-line. At sacrifice, 60 days after sensitization, animals were weighed and testes fixed and stained as above. Scoring of histological lesions includes minimal and maximal damage to both testes in accordance with the more complete index used by Bishop *et al.*(3). Sera, collected by cardiac puncture just prior to sacrifice, were tested for humoral antibody by passive cutaneous anaphylaxis (PCA)(14). PCA challenge was effected by testicular extract prepared according to the acetic acid-ammonium sulfate procedure of Freund *et al.*(4).

TABLE I. Germinal Lesions in Male Guinea Pigs Repetitively Injected with Testicular Antigen without Adjuvant.

Group	No. of animals	Strain	Testicular homogenate* ml/inj.	Inj./wk	Total inj.	Duration (days)	Germinal damage†
A	5	Mixed	.1	3	27-63	63-157	0, 3, 0, 4, 3, 4
B	6	”	.2	2	18-42	63-157	1, 3, 2, 4, 1, 2, 3, 3
C	5	Hartley	.1	3	9-47	21-109	2, 2, 3, 3, 3
D	6	”	.05	6	27-95	31-109	3, 3, 4, 4, 3, 3

* Approximately 250 mg (wet wt) testes/ml.
† One guinea pig in group A and 2 in B unilaterally orchiectomized at day 63.

Results. Repeated injections without adjuvant. Data for 22 guinea pigs injected repetitively with testicular homogenate alone are summarized in Table I. The most severe testicular responses occurred after 100 days in guinea pigs which had received 38, 54, 63, and 95 injections at rates of 3 or 6 times per week (Fig. 1, 2). Damage was discernible, however, within one month in some animals injected with 0.1 or 0.05 ml, 3 or 6 times per week, respectively. While not conclusive, the lower dosage (0.05 ml), frequently administered (6 inj/wk), tended to evoke the greatest response. In no case was germinal damage complete, such as that which frequently occurs when antigen is combined with adjuvant; on the contrary, lesions tended to be patchy and involved only one or several tubules. During the course of injections, it was observed that reinjection into the same area evoked a marked localized erythematous reaction which persisted for 1 to 2 days.

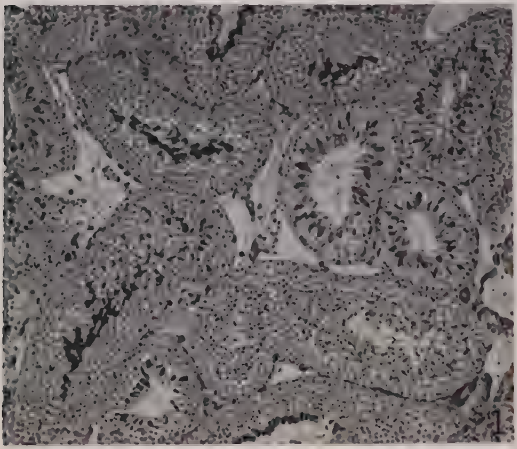
Antigen and adjuvant uncombined. Separate injections of testicular antigen and complete adjuvant quite readily elicited germinal responses, so long as both components were administered on the same side of the nuchal mid-line (Table II). Severe and almost complete destruction of germinal epithelium of both testes occurred in guinea pigs in which

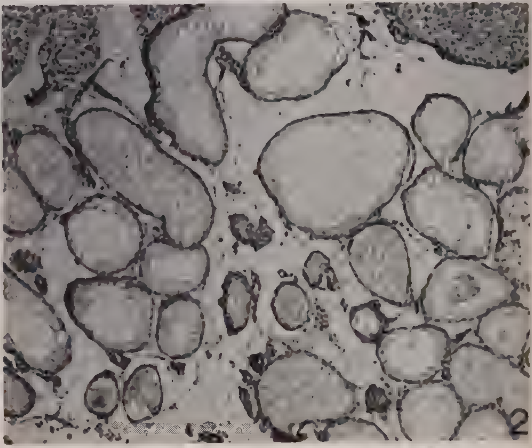
TABLE II. Aspermatogenesis Induced in Guinea Pigs Injected with Testicular Antigen (0.5 ml) and Complete Adjuvant (0.5 ml) in Separate Sites on Dorsal Quadrant.

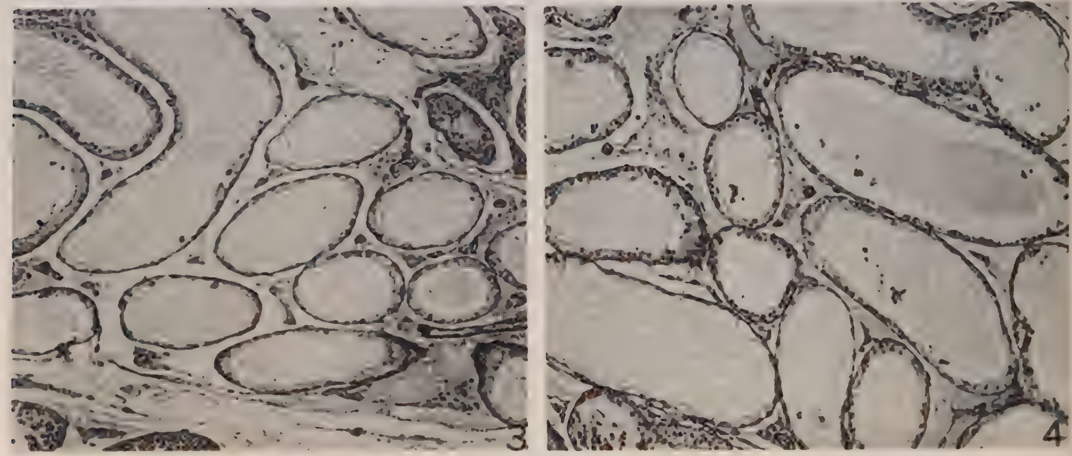
No.	Strain	Sites of inj. Antigen	Sites of inj. Adjuvant	Distance between inj. sites (cm)	Germinal damage Minimum	Germinal damage Maximum	PCA*
1	Mixed	Lateral	Median	3.5	0	3	
2	”	”	”	3.0	0	3	
3	”	”	”	3.0	0	3	
4	”	”	”	4.0	0	2	
5	”	Median	Lateral	3.5	0	3	
6	”	”	”	3.5	0	4	
7	”	”	”	4.0	0	3	
8	Hartley	”	”	2.5	0	4	—, —
9	”	”	”	3.0	1	4	+, +
10	”	”	”	2.5	2	4	+, —
11	”	”	”	2.8	2	4	+, +
12	”	”	”	2.7	3	4	+, +
13	”	”	”	2.5	0	3	+, +
14	”	Lateral	Median	2.5	0	3	+, +
15	”	”	”	2.5	0	3	—, —
16	”	”	”	2.5	0	4	+, +
17	”	”	”	2.0	2	4	+, +
18	”	”	”	2.4	3	4	+, +
19	”	”	”	2.8	3	4	+, +
20	”	Right	Left	6.0	0	1	—, —
21	”	”	”	6.0	0	2	+, —
22	”	”	”	6.0	0	1	—, —
23	”	”	”	6.0	0	1	+, —

* PCA tests run in duplicate: +, positive; —, negative response.

a minimal distance of 2.4 to 2.8 cm separated the sites (Fig. 3, 4) Aspermatogenesis was induced regardless of which component was medially injected; a slight edge seems to fa-







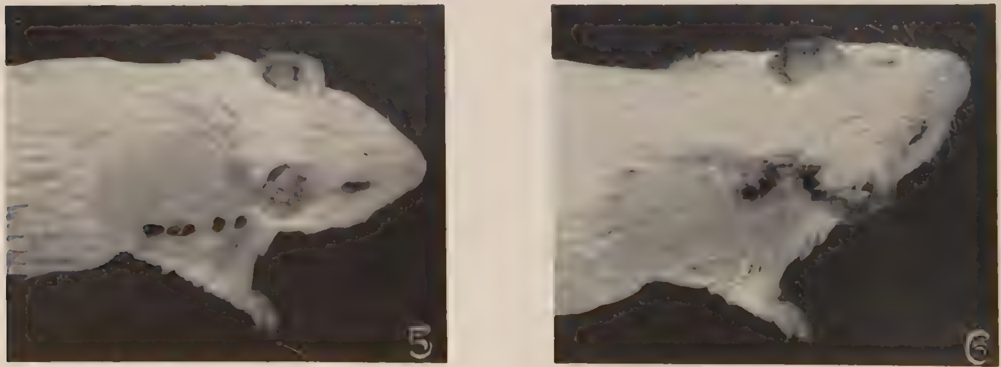


FIG. 1. Normal adult testis of donor guinea pig. × 65.

FIG. 2. Aspermatogenesis induced by testicular homogenate without adjuvant; 54 injections, 0.1 ml/inj, 3/wk. × 65.

FIG. 3-4. Severe germinal damage after separate-site injection of antigen and complete adjuvant; nos. 9 and 18, Table II. × 65.

FIG. 5-6. Granulomata at lateral and median sites of adjuvant injection in animals nos. 10 and 14, respectively (Table II).

vor those with medially administered antigen and lateral adjuvant. No histological abnormalities were detected in the testicular interstitium. The sensitizing injections were followed by granulomata at sites of adjuvant administration (Fig. 5, 6), reactions which are typical of animals treated with combined antigen-adjuvant mixtures(2).

Four control guinea pigs injected with antigen on the right and adjuvant on the left side of the mid-line were relatively free from germinal change (Table II). Depletion of sperm and sloughing of spermatocytes in the seminiferous tubules of one, however, indicated an induced response but the significance of this case is obscure.

PCA reactivity of sera of separate-site animals was generally positive (Table II). Two samples failed, by this method, to show circulating antibody. At the same time, sera of two other guinea pigs (Nos. 21, 23), injected on both sides of the mid-line, indicated the presence of antibody in one of 2 duplicate tests.

Discussion. These experiments show that aspermatogenesis can be induced in the guinea pig by testicular antigen without prior incorporation into adjuvant. When testis is frequently injected over a long period of time without adjuvant, the immune mechanism may be different from that when adjuvant is employed, but the ultimate tissue response appears to be similar. Although incomplete in these experiments, it is possible that total germinal destruction might occur if sufficient injections were made over an adequate period. The further possibility exists that in the absence of adjuvant, a constituent of the skin itself may play a role in mediation of the immune response(8,15). Aspermatogenesis, like experimental allergic encephalomyelitis(10), can thus be induced by maintenance of an adequate source of tissue antigen in appropriate sites.

Excellent germinal responses resulted from introduction of antigen and adjuvant in separate sites on the same side of the mid-line. This suggests that the lymphatic drainage system is involved, and that if there need be association of the 2 components, it must necessarily occur in, or be mediated by, the lymph nodes(11). The slight tendency for more severe responses to occur when adjuvant was injected laterally (Table II), where gross lymph nodes are more abundant, favors such an interpretation. Further attempts to test this experimentally have as yet proved unfruitful owing to the diffuse pattern of the guinea pig lymphatic system(16) and difficulties of ablation. The correlation of induced lesions in the 2 testes of a responsive animal confirms the phenomenon discussed more fully elsewhere(3).

The serological data are merely suggestive but support the contention that positive PCA reactions indicate an active immune state significantly related to the aspermatogenic response(3). Systemic anaphylaxis, as determined by the Schultz-Dale test, has also been associated with the aspermatogenetic syndrome(7), but for certain types of immune reactions, at least, this method is regarded as less sensitive than the PCA procedure(17). Active cutaneous sensitization can be induced in guinea pigs by injection of testicular antigen without mycobacterial adjuvant (4,5).

Summary. Aspermatogenesis was induced in adult guinea pigs (1) by repeated intracutaneous injections of homologous testicular homogenate without adjuvant, and (2) by injection of testicular antigen and complete adjuvant in separate sites on the same side of the nuchal mid-line. With testicular tissue alone, severe germinal lesions were induced after 100 days in animals injected 3 or 6 times weekly with 0.1 or 0.05 ml, respectively. Degree of germinal damage resulting from separate-site injection of uncombined antigen and adjuvant was comparable to that when testis is incorporated into complete paraffin oil adjuvants prior to administration. Results of separate-site injections suggest dependency on a common lymphatic drainage system.

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1. Freund, J., Lipton, M. M., Thompson, G. E., *J. Exp. Med.*, 1953, v97, 711.

2. Katsh, S., Bishop, D. W., *J. Embryol. Exp. Morphol.*, 1958, v6, 94.

3. Bishop, D. W., Narbaitz, R., Lessof, M., *Develop. Biol.*, 1961, in press.

4. Freund, J., Thompson, G. E., Lipton, M. M.,

J. Exp. Med., 1955, v101, 591.

5. Freund, J., *Adv. Tuberc. Res.*, 1956, v7, 130.

6. ———, *J. Allergy*, 1957, v28, 18.

7. Katsh, S., *Internat. Arch. Allergy Appl. Immunol.*, 1959, v15, 172.

8. Raffel, S., *Immunity, Hypersensitivity, Serology*, Appleton-Century-Crofts, Inc., 1953, New York.

9. Eisler, M., *Z. f. Immunitätsforsch.*, 1959, v117, 294.

10. Rivers, T. M., Sprunt, D. H., Berry, G. P., *J. Exp. Med.*, 1933, v58, 39.

11. Stone, S. H., Freund, J., PROC. SOC. EXP. BIOL. AND MED., 1959, v102, 140.

12. Bishop, D. W., *Anat. Rec.*, 1960, v137, 340.

13. Himes, M., Moriber, L., *Stain Technol.*, 1956, v31, 67.

14. Ovary, Z., *Progr. Allergy*, 1958, v5, 459.

15. Chase, M., *J. Allergy*, 1957, v28, 30.

16. Gyllensten, L., *Acta anat.*, 1950, v10, 130.

17. Bier, O. G., Siqueira, M., Beraldo, W. T., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 29.

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Effectiveness of DTPA in Removing Plutonium from the Pig.* (26553)

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(Introduced by L. Bustad)
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The superiority of diethylenetriaminepentaacetate (DTPA) over chelating agents previously employed for removal of internally deposited plutonium is attested by a number of reports of its effectiveness in small animals (1-5). Its effectiveness in the human has been reported by Norwood(6). To help fill the gap between the rather extensive data on the rat and the very limited experience with man experiments were performed employing miniature swine. These animals have a weight comparable to man, and appear from preliminary studies to metabolize plutonium in a manner similar to man(7). The experiments were designed to measure the effectiveness of DTPA when administered promptly following plutonium injection, and when administered after a long interval following plutonium injection.

Methods. The miniature swine employed were 13-15 month males of the Pitman-Moore strain, confined to metabolism cages throughout the experiment. In the prompt treatment experiment 6 animals weighing from 40 to 60 kg were injected intravenously with 25 μc of Pu^{239}, administered as the tetravalent citrate complex in pH 5 solution. One hour later, 3 of the animals were injected intravenously with 40 ml of a pH 7 solution containing 9 g of the calcium trisodium salt of DTPA. In the delayed treatment experiment two 15 month males weighing 62 and 75 kg, injected 2 months previously with approximately 175 μc of plutonium IV citrate, were given 5 consecutive daily intravenous injections of $CaNa_3DTPA$, one gram the first day and 2 g on each of the succeeding 4 days. Excreta were collected daily in both experiments; daily blood samples were obtained only in the delayed treatment experiment. All animals were sacrificed 6 or 7 days following treatment. Complete tissue analyses were performed on animals from the prompt treatment experiment.

Results of prompt treatment. Table I

TABLE I. Effect of Single Prompt Dose of DTPA on Excretion of Plutonium by Pigs.

	Excretion (% dose/day)			
	Urine		Feces	
Day post inj.	Control*	DTPA treated*	Control	DTPA treated*
1	.6	45.	.1	1.8
2	.1	29.	.3	1.3
3	.2	6.8	.4	1.7
4	.2	.5	.4	.4
5	.1	1.0	.2	.3
6	.1	.3	.1	.1
Total	1.3 (.9-1.9)	83 (78-88)	1.5 (1.2-2.0)	5.6 (3.5-8.9)

* Avg of results from 3 pigs (range in parentheses).

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* Work performed under Contract between Atomic Energy Comm. and General Electric Co.

TABLE II. Effect of Single Prompt Dose of DTPA on Retention of Plutonium by Pigs.

Tissue	Retention (% of dose/total tissue)			
	Control*		DTPA treated*	
Liver	14.	(11. –16.)	.47	(.32 – .55)
Kidneys	.20	(.16 – .27)	.20	(.19 – .20)
Spleen	.24	(.14 – .28)	.013	(.012 – .013)
Testes	.10	(.08 – .12)	.009	(.007 – .010)
Adrenals	.0017	(.0015– .0019)	.0005	(.0005– .0006)
Femur	2.5	(1.7 – 3.4)	.29	(.20 – .38)
Total skeleton	57.	(56.5 –57.6)†	5.9	(5.2 – 7.1)
Excreta: Urine	1.3	(.9 – 1.9)	83.	(78. – 88.)
Feces	1.5	(1.2 – 2.0)	5.6	(3.5 – 8.9)
Total recovered	74.	(70. –78.)†	96.	(93. –100.)

* Avg of results from 3 pigs (range in parentheses). † Data available from only 2 pigs.

shows average daily excretion of plutonium by the DTPA treated and control pigs. Enhanced excretion is evident predominantly in the urine, with the major effect during the first 3 days after treatment.

Table II shows results of tissue analyses on DTPA treated and control pigs. It is evident that DTPA exhibits some selectivity in its effect on tissue plutonium deposition. As previously observed in rats(*2*), concentration of plutonium in the liver is most favorably affected, while concentration in the kidney is least affected. The low recovery of only 74% of total injected plutonium in the case of the control animals is probably due to sampling problems, and to analytical difficulties in assay of the very large tissue samples involved. Such difficulties would have much less effect on total recovery in the DTPA treated animals where tissue plutonium content was lower by an average factor of about 10. While all tissue concentrations may therefore be low by as much as 25%, the general conclusions with regard to the effectiveness of DTPA treatment are not altered.

TABLE III. Excretion of Plutonium by Pigs Administered DTPA 60 Days after Plutonium Deposition.

Days since Pu inj.	DTPA admin. (g)	Pig 1 Urine (μc/day)	Pig 1 Feces (μc/day)	Pig 2 Urine (μc/day)	Pig 2 Feces (μc/day)
57		.02	.004		.004
58		.01	.003	.01	.005
59		.02	.004	.01	.005
60	1	.01	.003	.01	.005
61	2	1.2	.04	(lost)	1.3
62	2	1.3	.8	1.4	.8
63	2	.7	1.7	2.3	3.8
64	2	.8	3.9	2.1	4.1
65	1	1.2	1.5	2.8	.2
66		.2	.9	.7	2.6
67		.3	1.5	.5	5.4
68		.3	.4	.10	.8
69		.2	.4	(lost)	2.3
70		.15	(lost)	.2	5.6
71		.14	(lost)	.5	(lost)
Total excretion (μc)		6.6	11.2	10.6	26.9
Total excreted (% of initially inj. dose)		4	7	5	14

Results of delayed treatment. Table III shows the data on plutonium excretion of 2 pigs for 3 days prior to treatment, for 5 days during treatment, and for a subsequent 7-day period following treatment. The pretreatment data serve as a control for the treatment period and indicate an increase in plutonium excretion during and following the treatment period by factors of approximately 40 and 100 in the urine, and factors of 300 and 500 in the feces. Total plutonium recovered in the excreta represents in the one case 11% and in the other case 19% of the plutonium injected 60 days prior to treatment. Of particular interest is the fact that the effect of treatment was evident for at least 7 days following cessation.

Daily blood analyses showed plutonium contents ranging from 12-46 μμc/g with no evidence of a correlation between plutonium level and time of DTPA administration.

Discussion. The enhanced excretion of plutonium obtained through both prompt and delayed treatment with DTPA is clearly of a magnitude to recommend consideration of such treatment in cases of human exposure to plutonium. Reduction of plutonium deposition in bone by a factor of 10, and in liver by a factor of 30, as a consequence of treatment one hour following plutonium injection is a more dramatic observation than the 11 or 19% removal of plutonium occasioned by the delayed treatment. The effectiveness of the delayed treatment is perhaps the more surprising, and the more encouraging of the 2 observations, however, for the delayed treatment was successful in removing plutonium which had been firmly fixed in tissues whereas the plutonium removed by the prompt treatment was probably still present in large amount in the blood stream. Also, the DTPA dosage employed in the delayed treatment was a conservative one, comparable to those which have been employed without untoward effects in man(6,8); while the single dose administered in the prompt treatment experiment was somewhat larger than would probably be considered appropriate for man.

The difference in pattern of plutonium excretion in the 2 experiments is particularly noteworthy. With prompt treatment, increased excretion is primarily *via* the urine, while delayed treatment results in large increases in both urinary and fecal excretion, with fecal excretion predominant. One may speculate that the fecal plutonium is that which is removed *via* the bile from liver. This is consistent with observations of delayed DTPA treatment of plutonium bearing rats, where more than three-fourths of the plutonium in the liver was removed, and only about one-fourth of that in bone removed(2). A reciprocal relationship between plutonium appearing in the feces and that retained in the liver of rats during treatment with DTPA has also been noted by Fried *et al.*(9). The predominance of fecal excretion of plutonium was not observed by Norwood in his human cases(6). These, however, were treated at much longer intervals following plutonium deposition.

An encouraging finding was that of the sustained action of DTPA during the week following delayed treatment. Urinary excretion of plutonium decreased rather abruptly upon cessation of treatment, but remained a factor of 10 or more higher than pretreatment levels. Fecal excretion of plutonium during the week following treatment was nearly as high, or in some cases actually higher than during the treatment period. Substantial additional amounts of plutonium might have been excreted had the animals not been sacrificed when they were. Lindenbaum and Schubert have recently shown that the less pronounced sustained effects of DTPA treatment in rats are a reflection of an increased ultrafiltrability of the tissue deposited plutonium(10). This sustained effect of DTPA has also been noted by Norwood in man and is much more pronounced than in the case of treatment with EDTA(6). As a hypothesis one might propose that the sustained elimination of plutonium occurs principally from the liver and may be due to: (1) ability of DTPA (to a greater extent than EDTA) to penetrate the reticulo-endothelial cells in which the plutonium is retained as a colloid-sized aggregate, and (2) slow action of this intracellular DTPA in solubilizing these plutonium aggregates. In support of such a hypothesis, Foreman, using labeled chelating agents, has shown that a small fraction of administered DTPA is retained for long periods and that this fraction is substantially greater than the fraction of EDTA which is similarly retained(5). Schubert and Fried have shown in experiments with rats that thorium when administered in ionic form is more promptly removed by DTPA treatment than when administered in colloidal form (11). The colloidal thorium is deposited predominantly in the reticulo-endothelial system and its excretion occurs only gradually and at an increasing rate as treatment is continued over a period of several days.

The observation during the delayed DTPA treatment that blood levels of plutonium were not increased, despite, on certain days, an increase of a hundred-fold or more, in rate of urinary excretion of plutonium, remains unexplained. Quite possibly the chelated plutonium which reaches the blood from the tissues is so rapidly excreted that it exerts little influence on total blood plutonium levels, even though it may, in total quantity, greatly surpass the amount of plutonium in the blood at any given moment. The further study of blood plutonium levels in relation to administration of chelating agents would seem to offer much promise of enlightenment with regard to the mechanisms involved in the therapeutic effectiveness of these agents.

Summary. A single intravenous dose of diethylenetriaminepentaacetate (DTPA) one hour after plutonium administration resulted in excretion of approximately 90% of the plutonium. As compared with untreated controls, retention of plutonium in the skeleton was reduced by a factor of 10 and retention in the liver was reduced by a factor of 30. Two pigs treated with 5 consecutive daily injections of DTPA excreted 11 and 19% of the plutonium injected 60 days prior to treatment.

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1. Smith, V. H., *Nature,* 1958, v181, 1792.
2. ———, *Hanford Biology Research-Annual Report for* 1958, HW-59500, 1959, 63.
3. Fried, J. F., Graul, E. H., Schubert, J., Westfall, W. M., *Atompraxis,* 1959, v5, 1.
4. Rosenthal, M. W., *Elimination of Radioisotopes from the Body* in *Radioisotopes in the Biosphere,* Univ. of Minnesota, Minneapolis, 1960, 541.
5. Foreman, H., Application of Chelating Agents for Hastening Excretion of Radioelements, in *Metal Binding in Medicine,* J. B. Lippincott Co., in press.
6. Norwood, W. D., *J. Occupational Med.,* 1960, v2, 371.
7. Clarke, W. J., McKenney, J. R., Horstman, V. G., Seigneur, L. J., Terry, J. L., Bustad, L. K., *Hanford Biology Research-Annual Report for* 1958, HW-59500, 1959, 54.
8. Spencer, H., private communication.
9. Fried, J. F., Lindenbaum, A., Rosenthal, M. W., unpublished results cited in reference(4).
10. Lindenbaum, A., Schubert, J., *Nature,* 1960, v187, 575.
11. Schubert, J., Fried, J. F., *ibid.,* 1960, v185, 551.

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Histidine Decarboxylase Activity of Basophils from Chronic Myelogenous Leukemic Patients. Origin of Blood Histamine.* (26554)

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It is well established that relatively large amounts of histamine are present in tissue mast cells(1-3), and leukocytes(4-6), especially the granulocytes and probably largely the basophils(4,7).† Eosinophils have also been claimed to contain histamine(7).

A polemic has existed since the first report of Code in 1936(4) regarding the origin of histamine in the body. Some argued that it originated solely from ingestion with food and from decarboxylation of histidine by intestinal flora, and others asserted that it also originated by the action of a tissue decarboxylase. The bulk of evidence supports the concept that tissue mast cells of mammalian organisms decarboxylate histidine to histamine(1-3,11) although some workers have denied this(8). Only a few investigators have ruled out bacterial decarboxylation in their *in vitro* studies. These are Werle, *et al.*(12) who used sterile precautions and tested for contamination after incubation; Gustafsson, Kahlson and Rosengren who worked with germ-free rats(9); and Schindler, Day and Fisher who worked with sterile

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† Whether or not the blood basophils are related to mast cells is not settled.

tissue cultures of malignant murine mast cells (11).

No one has demonstrated that a human tissue can synthesize histamine in the proved absence of bacteria, nor has the origin of blood histamine been elucidated. Schayer and Kobayashi(10) showed that unsterile rabbit blood platelets convert about 1.3% C^{14}-histidine to C^{14}-histamine, but the rabbit is the only species known which has a high concentration of histamine in the platelets, although histaminase may be present in the absence of histamine in the platelets of other species. For these reasons we attempted to demonstrate the ability of a sterile human cell, the blood basophil, to convert C^{14}-histidine to C^{14}-histamine.

Methods. The only practical way to obtain concentrated human blood basophils seemed to be to use the buffy layer from patients with chronic myelogenous leukemia and very high basophilia. Such cases are rare. The literature has been well documented recently(13). After a protracted search, 3 cases were found over a period of several years. Studies on the first were done with unlabeled histidine and the results were equivocal. The other 2 yielded satisfactory results with radioactive histidine.

To strengthen the evidence that the basophils alone synthesized histamine, buffy layers predominating in other types of leukocytes were studied as well. These included lymphocytes from a case of chronic lymphatic leukemia, myeloblasts from 2 acute myelogenous leukemic patients, and eosinophils from one case of parasitic infestation and 2 cases of non-specific drug reaction. Platelets from a case of polycythemia vera also were studied.

In each case, 25 ml of blood were drawn under sterile conditions, and immediately added to an equal volume of a solution containing 5% polyvinylpyrrolidone and 0.5% trisodium citrate(14). The erythrocytes were allowed to sediment for 30-60 minutes, and the supernatant plasma containing the leukocytes was drawn off with a syringe and centrifuged at 29,000 × G for 10 minutes. The plasma layer was removed and 0.1-0.2 ml of the wet packed cells washed once with isotonic Tyrode's solution, recentrifuged, transferred to a Potter-Elvejhem glass homogenizer (Kontes Conical Type) and homogenized in 1 ml ice cold distilled water.

The homogenates were incubated with 16 μg (in 100 λ) of L-histidine-2-(ring)-C^{14} (California Corp. for Biochemical Research, Los Angeles) containing 0.3 μC or 200,000 counts per minute (CPM) at infinite thinness in the Nuclear-Chicago low-background flow counter. Forty micrograms of pyridoxal-5-phosphate (Merck) in 2 ml of 0.1 M potassium phosphate buffer, pH 7.4, were added as cofactor. Aminoguanidine, 100 μg was added to inhibit diamine oxidase. The incubation was carried out in the Dubnoff Metabolic Incubator at 37°C for 3 hours. Controls were run in which the enzyme was inactivated with heat or 2 drops of concentrated perchloric acid. All solutions and glassware were sterile, and strictly sterile precautions were observed throughout, transfers being made under ultra-violet lamps. At the end of all incubations, aliquots of all flasks were tested for the absence of bacteria by streaking on conventional blood-agar plates. Occasional cultures were made in tubes of standard media. The results on the rare samples which showed contamination were discarded.

The labeled histamine formed was isolated as the picrate by the isotopic dilution method of Schayer, Davis and Smiley(15), plated out on planchets and counted at infinite thickness in a windowless counter on an automatic planchet changer (Tracerlab and Nuclear-Chicago).

Results are summarized in Table I. Platelets obtained from a case of polycythemia vera with a platelet count of 500,000, were isolated by a modification of the method of Klein, *et al.*(16), and similarly studied. No radioactive histamine was formed.

The results clearly show that only the buffy layers from the patients with basophilic leukemia formed histamine. In the second case, which gave 717 CPM (7170 at infinite thinness), the amount of histamine formed was estimated to be about 0.5 μg, representing about a 3% yield from the histidine added.

It would of course be desirable to obtain

TABLE I. Histidine Decarboxylase in Various Types of White Cells from Leukemic Patients.

Exp.	W.B.C.	Bas., %	Lympho., %	Eos., %	"Pro cells" and myeloblasts	Others, %	cpm*
N	15,100	77	5	5	0	10.0	415
S	71,370	49	3	7	2	39	717
B	301,000	0	98	0	0	2.0	0
Ba	54,000	0	0	0	99	1.0	2
R	30,000	0	—	43	—	57	1
O	13,000	0	49	28	0	23	1
M	15,000	0	—	70	—	30	0
Ch	15,400	0	22	0	78	0	0

* Counts after subtraction of cpm of identical acid killed blanks. The blanks were consistently about 20 cpm above a background of 2-5 cpm in the Nuclear-Chicago counter. The few samples run on the windowless Tracerlab counter also were 20-30 cpm above a background which usually averaged 25 cpm.

sterile basophils in pure form, possibly by modifications of the methods used for isolating mast cells, or by means of culturing them if possible. Nevertheless, the data offer an explanation for the origin of blood histamine other than that which might be absorbed from foods such as cheese, lobster, meat, etc., absorbed after decarboxylation of histidine by bacterial flora, or released from other sites in the body such as mast cells and gastric mucosa, although this is unlikely under most normal conditions.

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1. Wolstenholme, G. E. W., O'Connor, C. M. (Eds.), *Histamine*, Little, Brown and Co., Boston, 1956.

2. Fulton, G. P., Maynard, F. L., Riley, J. F., West, G. B., *Physiol. Rev.*, 1957, v37, 221.

3. Riley, J. F., *The Mast Cells*, E. & S. Livingston, Ltd., Edinburgh and London, 1959.

4. Code, C. F., *J. Physiol.*, 1936, v88, 10P.

5. Weidmann, H., Bucher, K., *Helv. Physiol. Pharmacol. Acta.*, 1951, v9, 87.

6. Lowry, O. H., Graham, H. T., Harris, F. B., Priebat, M. K., Marks, A. R., Bregman, R. V., *J. Pharmacol. Exp. Therap.*, 1954, v112, 116.

7. Code, C. F., Mitchell, R. G., *J. Physiol.*, 1957, v136, 449.

8. Waton, N. G., *Brit. J. Pharmacol. Chemotherap.*, 1956, v11, 119.

9. Gustafsson, B., Kahlson, G., Rosengren, E., *Acta Physiol. Scand.*, 1957, v41, 217.

10. Schayer, R. W., Kobayashi, Y., Proc. Soc. Exp. Biol. and Med., 1956, v92, 653.

11. Schindler, R., Day, M., Fischer, G. A., *Cancer Res.*, 1959, v19, 47.

12. Werle, E., Herrmann, H., *Biochem. Z.*, 1937, v291, 105.

13. Quattrin, N., Dini, E., Palumbo, E., *Blut*, 1959, v5, 166.

14. Maupin, B., *Rev. de Hématol.*, 1959, v14, 250; 355; 443.

15. Schayer, R. W., Davis, K. J., Smiley, R. L., *Am. J. Physiol.*, 1955, v182, 54.

16. Klein, E., Arnold, P., Earl, R. T., Wake, E., *New Eng. J. Med.*, 1956, v254, 1132.

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Gas-liquid Chromatography of Human Bile Acids.* (26555)

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Recent results from different laboratories (1,2,3) indicate that most of the biologically important steroids may be identified and quantitated with the aid of gas-liquid chromatography (GLC). VandenHeuvel, Sweeley and Horning(4) describe the separation of some bile acids and sex hormones with the aid of GLC and their technic offers a new way to a better understanding of the metabolism of steroids and diseases related to these compounds.

In the present paper, separation of bile acids from human bile with the aid of GLC has been evaluated and preliminary results are reported on the quantitative relationship between the major bile acids in man in some different conditions.

Methods. Bile was collected by aspiration into ethanol from the gallbladder of patients without hepatobiliary disease and also from patients with gallstones and biliary tract disease. Samples of duodenal contents were also collected through polyvinyl tubing introduced nasally. The tubing was introduced in the evening and the sample collected the next morning after intravenous injection of purified preparation of pancreozymin-cholecystokinin, ("Cecekin," obtained from Vitrum, Sweden). All samples were frozen and kept at –16°C.

As a rule 5 ml of the bile and the intestinal contents were extracted with 20 volumes of ethanol, filtered and evaporated to almost dryness *in vacuo*. Water was added to the concentrate and mixed with ethyleneglycol and 4 N sodium hydroxide in the proportions water + bile : ethyleneglycol : sodium hydroxide 2 : 1 : 1, and this mixture was refluxed on a sand bath for 24 hours. After hydrolysis and extraction with ether, the bile acids were partitioned 4 times between 30 ml of petroleum ether and 50 ml of 70% ethanol. The bile acids were then dissolved in methanol and methylated with diazomethane. The methyl esters of the bile acids were then dissolved in cyclohexanol and taken up with a micropipette.

Conditions for Gas-liquid chromatography: Pye Argon Chromatograph.

Column dimensions: 1300 × 4 mm I.D. straight glass tubing.
Solid support Celite (80/100 mesh), acid washed, alkali treated.

Stationary Phase: Silicone Elastomer, E. 301 I.C.I. Lim. Nobel Division, Glasgow, England, Stationary phase 12% by weight of solid support.

Temperatures: Injection 280°C. Column and detector 245°C.

Carrier gas: Argon at 70 ml/min. Inlet pressure ca. 1.5 atm.

Outlet pressure: atm.

Detector: Beta ionization detector at 1,000 V.

Recorder: Philip recorder, 1 volt full scale.

Sample size: 0.5 μl, 5% solution in cyclohexanol.

Analysis time: ca. 45 min.

The response of the beta ionization detector is primarily a function of molecular weight with argon as carrier gas. Under the conditions used there was a tendency toward somewhat lower values for the slower-moving trihydroxy bile acids and correspondingly high values for the faster moving monohydroxy bile acids. It was therefore necessary to calibrate the instrument with a test mixture of known composition and use the correction factor obtained for the peak areas in order to calculate the relative distribution of the bile acids in human bile.

Results. Fig. 1 illustrates the separation of a test mixture of lithocholic, deoxycholic, chenodeoxycholic, hyodeoxycholic and cholic acid. A good separation was obtained between the 3 dihydroxy bile acids. For clini-

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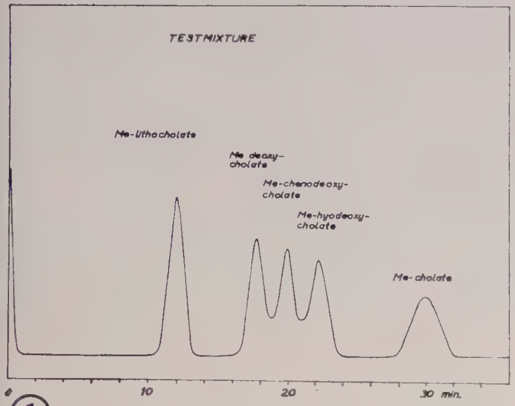

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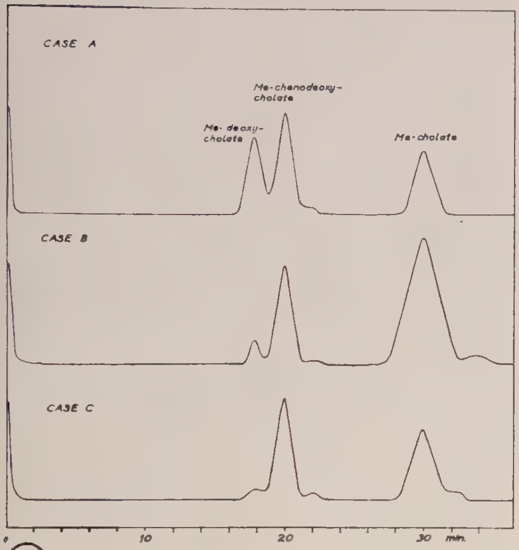

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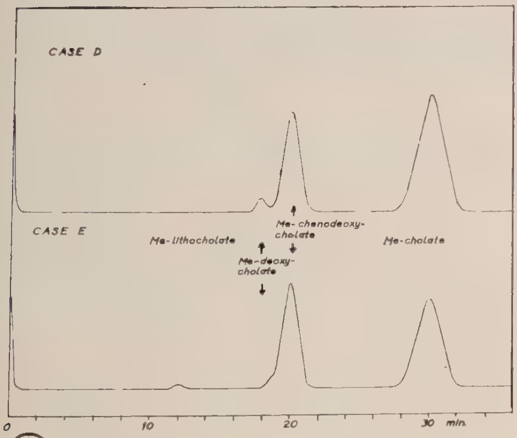

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TABLE I. Bile Acids in Gall-Bladder Bile of Patients with Different Diseases of Liver and Biliary Tract. In patient D hepatic bile was analyzed. In patient E duodenal content was analyzed for bile acids.

Patient	Sex	Age	Diagnosis	Ratio, C : CD : D
A	♂	46	Duodenal ulcer	.9 : 1 : .7
B	♀	37	Cholelithiasis	2.8 : 1 : .2
C	♂	45	Common duct stone with subsiding jaundice	1.1 : 1 : .2
D	♀	75	Cholelithiasis; choledochostomy performed; drainage bile analyzed	1.9 : 1 : .1
E	"	67	Familial hypercholesterolemia; free diet	1.1 : 1 : .02
F	"	53	Cholelithiasis	1.1 : 1 : 2.2
G	♂	62	"	.5 : 1 : 1.1

cal application of this technic it might be valuable to add hyodeoxycholic acid as internal standard both for qualitative and quantitative determinations. The results are very reproducible if all conditions are properly standardized.

In Fig. 2 some gas chromatograms from different patients are shown. A is a normal patient, case B is a patient with gallstones and a normal gall-bladder and case C is a patient with gallstone and subsiding jaundice. Apparently there is variation in the proportions of the different bile acids, which is most pronounced in the concentration of deoxycholic acid. In case B and C some unidentified bile acids are also present on the gas-chromatogram.

FIG. 1. Elution curve of a known mixture of methyl esters of bile acids. The stationary phase was a silicone rubber gum E. 301 maintained at 245°C on a 4-foot column. Flow rate was 70 ml argon per min. Samples were injected in a cyclohexanol solution. Recorder sensitivity 1 volt, full scale.

FIG. 2. Gas chromatogram of bile acid methyl esters in human gall-bladder bile. Operating conditions as described in Fig. 1, Case A. Gall-bladder bile acid methyl esters from a patient without known hepatobiliary disease. Case B. Gall-bladder bile acid esters from a patient with cholelithiasis but normal gall-bladder function. Case C. Gall-bladder bile acid esters from a patient with a common duct stone and subsiding jaundice.

FIG. 3. Case D. Gas chromatogram of bile acid methyl esters in hepatic bile from a patient without jaundice. Case D. Gas chromatogram of bile acid methyl esters in intestinal content of a patient with hypercholesterolemia.

In 2 patients hepatic bile was analyzed and in both patients only traces of deoxycholic acid were found. One of these chromatograms is given in Fig. 3 case D. Case E in Fig. 3 is a patient with familial hypercholesterolemia. The 2 major bile acids were chenodeoxycholic acid and cholic acid. Only traces of deoxycholic acid were found in this patient together with small amounts of lithocholic acid.

Conclusions. The major bile acids in samples of bile as determined with the aid of gas-liquid chromatography were cholic acid, chenodeoxycholic and deoxycholic acid. Regarding the proportions of the different bile acids, the results from this small group agree with those of Wootton and Wiggins(5), Isaksson(6) and Sjövall(7,8). Deoxycholic acid seemed to disappear in obstructive jaundice. In hepatic bile obtained from patients without jaundice only very small amounts of deoxycholic acid were found. Similar results have been reported by Sjövall(7) and Rudman and Kendall(9).

An interesting finding is the very small amount of deoxycholic acid present in a patient with familial hypercholesterolemia. This patient had been on a general mixed diet without the consumption of highly unsaturated fats or any other treatment of the hypercholesterolemia. Sjövall(7) has reported a similar bile acid pattern in a patient with hypercholesterolemia but on a formula diet. Further studies are in progress on the bile acid pattern in diseases with an altered cholesterol metabolism.

The author wishes to thank colleagues at the Dept. of Surgery for samples of bile. Technical assistance of Mr. J. Gürtler is gratefully acknowledged.

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1. Beerthuis, R. K., Recourt, J. H., *Nature,* 1960, v186, 372.
2. VandenHeuvel, W. J. A., Horning, E. C., *Biochem. Biophys. Res. Com.,* 1960, v3, 356.
3. Sweeley, C. C., Horning, E. C., *Nature,* 1960, v187, 40.
4. VandenHeuvel, W. J. A., Sweeley, C. C., Horning, E. C., *Biochem. Biophys. Res. Com.,* 1960, v3, 33.
5. Wootton, I. D. P., Wiggins, H. S., *Biochem. J.,* 1953, v55, 292.
6. Isaksson, B., *Acta Soc. Med. Upsaliensis,* 1953-54, v59, 307.
7. Sjövall, J., *Clin. Chem. Acta,* 1960, v5, 33.
8. Encrantz, J. C., Sjövall, J., *Acta Chem. Scand.,* 1957, v11, 1093.
9. Rudman, D., Kendall, F. E., *J. Clin. Invest.,* 1957, v36, 530.

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Effect of Citrus Bioflavonoids on Metabolism of Hydrocortisone in Man.* (26556)

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It has been recognized for some time that the class of compounds generally termed bioflavonoids may be of physiological importance(1,2). Our previous observations of the relatively transient rises in plasma 17-hydroxycorticosteroid levels following major elective surgery(3) have interested us in possible pharmacological agents that would decrease the rate of metabolic inactivation, *i.e.,* Δ^4-3-keto reduction, or, due to other factors, impede removal from plasma of hydrocortisone and possibly other adrenocortical steroids. Masri and DeEds(4) observed that administration of certain bioflavonoids to rats produces involution of the thymus gland in the presence of the adrenal glands, but *not* after their removal, suggesting mediation of the physiological effects of bioflavonoids through an action on the pituitary-adrenal axis. It occurred to us that these findings might be in part explained on the basis of prolongation of the biological half-life of the rat's endogenously produced corticosterone and that the bioflavonoids may have acted in a similar manner to salicylic acid(5,6) and possibly N-acetyl-p-aminophenol (NAPA) as suggested by Corte and Johnson(7) in connection with the observed prolongation of the half-life of hydrocortisone in rabbits following administration of NAPA.

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We believe that a non-toxic pharmacological agent which could prolong the biological half-life, and thereby activity, of endogenous and exogenous hydrocortisone might be of clinical importance. This report concerns the effects of various bioflavonoids on the metabolism of hydrocortisone in normal man.

Materials and methods. The patients who participated in these studies were volunteers of both sexes. All had been hospitalized for at least 3 weeks following surgical repair of fractures of long bones or recovery from knife wounds not involving the abdominal cavity. They were in an adequate nutritional status with no laboratory evidence of metabolic disorder. To 19 such subjects we have administered parenterally 50 and 100 mg doses of Hydrocortone®—Merck,[†], an intravenous infusion concentrate of hydrocortisone which was diluted with 250 ml of 5% Dextrose in water and infused over a period of 15 minutes. Fasting venous blood samples (8:00 a.m.) were obtained on several days before the infusion, and 15, 30, 60, 120, 180, 240 and 360 minutes following the end of infusion of hydrocortisone. This procedure was repeated after several days with the inclusion of certain bioflavonoids in the infusion mixture, or in identical fashion following a period of 6 or 7 days of oral administration of the bioflavonoids.

In several instances no hydrocortisone was administered but endogenous hydrocortisone levels in plasma were measured once daily during oral administration of the bioflavonoids. Tracer amounts of 4C^{14}-hydrocortisone were similarly employed in several observations. Bioflavonoids used were Hesperetin, Eriodictyol glycoside and Hesperidin methyl chalcone. Hesperetin[‡] was formulated as a suspension in glucose syrup slightly flavored with oil of orange, N.F., so that 15 ml of suspension contained 2 g of hesperetin. The Eriodictyol glycoside was administered as a sterile solution added to 250 ml of 5% Dextrose in water. Hesperidin methyl chalcone was also formulated as a solution in glucose syrup flavored with natural oil of lemon and limes, so that 25 ml of solution contained 1.5 g of hesperidin methyl chalcone. N-acetyl-p-aminophenol (NAPA)[§] was administered by mouth in tablet form. "Free" and conjugated 17-hydroxycorticosteroids were measured by a modified procedure based on the authors' own methods(8). Following administration of 4C^{14}-hydrocortisone, total radioactivity in the free and conjugated plasma fractions were determined by counting in an alcohol-toluene, DPO (diphenyloxyzol) and POPOP (1,4-bis-2(5-phenyloxazolyl)-benzene) phosphor in a Packard liquid scintillation spectrometer.

Results. The logarithmic values of the plasma "free" 17-OH corticosteroid concentrations, measured as Porter-Silber chromogens, are plotted against time and a linear relationship is obtained one hour after the end of infusion of hydrocortisone. From such curves the biological half-life of the infused steroid has been calculated(9). In all cases herein presented an increase of this half-life has been consistently observed following oral or parenteral administration of various bioflavonoids and of NAPA (Table I). Maximal concentrations of "free" 17-OHCS following hydrocortisone infusion are not affected by administration of these compounds. Rates of disappearance (Fig. 1) from the plasma of normal subjects before and after administration of bioflavonoids and NAPA

[†] Hydrocortone® was generously supplied by Merck, Sharp & Dohme, Research Laboratories, West Point, Pa.

[‡] Hesperetin, preparations 59-S-1 and C-7090, Eriodictyol glycoside from lemon, preparations P-56 and P-57 and Hesperidin methyl chalcone were supplied by Sunkist Growers, Inc., Ontario, Calif.

[§] Kindly supplied to us by F. W. Horner, Ltd., Montreal, Canada under the trade name of "Atasol".

TABLE I. Effect of Various Bioflavonoids and NAPA* on Half-Life of Intravenously Administered Hydrocortisone.

No. of subjects	Ref. letters (Fig. 1)	Hydrocortisone, mg	Compound administered			Max conc. "free" 17-OH CS in plasma, $\mu g\ \%$	t ½ (9), min.	"t" test P
			Compound	Amount	Duration			
2		3 μc $4C^{14}$					94†	
2		3 μc $4C^{14}$	Hesperetin	2 g t.i.d.	7 days		148	
3		50				72†	99	
3		"	"	2 g t.i.d.	7 days	97	207	
14	A	100				165 ± 8‡	98 ± 2.6‡	
2	B	"	Eriodictyol	50 mg I.V.	15 min.	170	135	
2	C	"	Hesperidin methyl chalcone	100 mg I.V.	15 min.	185	150	
3	D	"	Hesperetin	2 g t.i.d.	7 days	160 ± 7.1	162 ± 6.3	$.01 < P < .05$
2	E	"	NAPA*	2 g t.i.d.	6 days	145	168	
5	F	"	Hesperidin methyl chalcone	1.5 g	6 days	178 ± 7.4	184 ± 19.5	$.01 < P < .05$

* N-Acetyl-p-Aminophenol (Atasol, Horner). † Mean values. ‡ S.E.

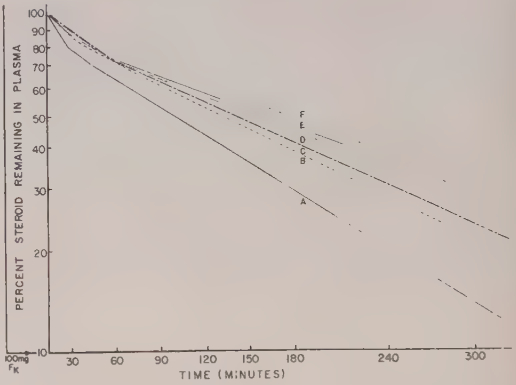

FIG. 1. Disappearance of hydrocortisone from plasma following intravenous administration. (See Table I for explanation of letters A through F.)

are presented in a manner similar to Peterson(10). The delayed disappearance of hydrocortisone following administration of these compounds together with the resulting half-life (t ½) values suggests that an altered metabolic rate of the infused hydrocortisone results from the bioflavonoids and NAPA or their respective metabolites. Examples of the effects of bioflavonoid administration on the endogenous 17-OHCS levels in plasma (Table II) indicate a similar alteration of the metabolic rate of endogenously produced 17-OHCS. This is confirmed by the results obtained with the tracer amounts of $4C^{14}$-hydrocortisone (Table I).

The values in Tables I and II are mean values obtained from the indicated number of observations. Whenever possible, standard errors of the mean were calculated and the data subjected to a "t" test of significance of the difference in the half-life of hydrocortisone obtained before and after administration of the various compounds. The resulting P values are given.

Discussion. These studies indicate that steroid degradation, judging from rates of disappearance of the 17-OHCS in plasma, is significantly decreased following administration of certain non-toxic phenolic compounds, such as the bioflavonoids hesperetin, eriodictyol and hesperidin methyl chalcone, and the drug N-acetyl-p-aminophenol. More data are needed to explain the mechanisms involved in these effects. Corte and Johnson(7) have suggested that NAPA is a competitive "glucuronylation" inhibitor of steroids. Our preliminary studies of the glucuronic acid conjugated 17-OHCS in plasma, following infusion of hydrocortisone discount this hypothesis because there are no significant differences between the conjugated 17-OHCS levels before and after administration of these

TABLE II. Effect of Two Bioflavonoids and NAPA on Fasting (8 A.M.) "Free" 17-OH CS Levels in Plasma.

	Compound admin. (orally)		Control determinations (*i.e.* before admin. of drug)		μg % 17-OH CS (mean values)						
					Day of admin.						
No. of subjects	Compound	Amount	No. of det.	μg % 17-OH CS	1	2	3	4	5	6	7
4	Hesperetin	2 g t.i.d.	12	11 ± .44‡	12	12	14	17	19	20*	19
4	Hesperedin methyl chalcone	1.5 g q.i.d.	12	14 ± .48	17	21	24†	23	23	22	--
2	N-Acetyl-p-Aminophenol (NAPA)	2 g t.i.d.	6	13 ± .67	15	15	18	18	21	22	20

* Maximum value on 6th day: D.F. = 3; $t = 3.70$; $.01 < P < .05$.
† " " " 3rd " : D.F. = 3; $t = 3.36$; $.01 < P < .05$.
‡ Mean ± S.E.

compounds. Similarly a comparison of urinary 17-OHCS excretion during these 3 control days and the days of drug administration has not shown significant differences, in either "free" or conjugated fractions. The delayed disappearance of 17-OHCS from plasma would seem to be due to either a delayed reduction of the steroid, or other hitherto unknown factors impeding its removal. Use of $4C^{14}$– and $1,2H^{3}$– hydrocortisone and actual paper-chromatographic isolation of free labeled materials is contemplated in order to show definitively the prolongation of the half-life of unmetabolized hydrocortisone.

Summary. Administration to normal humans of certain phenolic compounds, such as citrus bioflavonoids and N-acetyl-p-aminophenol (NAPA) significantly decreased the rate of disappearance from the plasma of parenterally administered hydrocortisone.

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1. Shils, M. E., Goodhart, S. R., The flavonoids in biology and medicine. Monograph of Nat. Vit. Foundation, Inc., New York, Jan., 1956.

2. Miner, R. W., *Ann. N. Y. Acad. Sci.,* 1955, v61, 637.

3. Elman, R., Weichselbaum, T. E., Moncrief, J. C., Margraf, H. W., *A.M.A. Arch. Surg.,* 1955, v71, 697.

4. Masri, M. S., DeEds, F., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 707.

5. Peterson, R. E., Black, R. L., Bunim, J. J., *Arthritis and Rheumatism,* 1958, v1, 29.

6. Done, A. K., Ely, R. S., Kelley, V. C., PROC. SOC. EXP. BIOL. AND MED., 1956, v93, 294.

7. Corte, G., Johnson, W., *ibid.,* 1958, v97, 751.

8. Weichselbaum, T. E., Margraf, H. W., *J. Clin. Endocrinol. and Metab.,* 1955, v15, 970.

9. Peterson, R. E., Wyngaarden, J. B., Guerra, S. L., Brodie, B. B., Bunim, J. J., *J. Clin. Invest.,* 1955, v34, 1779.

10. Peterson, R. E., *ibid.,* 1958, v37, 736.

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Effect of Adrenocortical Steroids on Bone Electrolyte Metabolism.* (26557)

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Bone contains about 40% of the total body sodium. It therefore has the potential of being an important factor in electrolyte metabolism.

Harrison(1) concluded from studies on normal rats and rats with various disorders of calcification, that bone sodium varies with calcium and that the calcium:sodium ratio in bone remains constant. Bergstrom and Wallace(2) subsequently demonstrated that bone sodium could vary independently from the calcium in acidosis associated with peritoneal dialysis(2,3,4,5) and following dietary salt restriction(2,3).

Studies of the role of the adrenal gland in bone electrolyte metabolism are conflicting. Adrenalectomy has been reported to cause bone sodium depletion in presence of a normal diet(6,7) a low salt diet(8) or with salt added to the drinking water(9). Other investigators, however, have reported that adrenalectomy failed to change bone sodium when combined with either a normal diet(10) or with salt added to drinking water(8).

Workers investigating adrenal insufficiency by balance studies in dogs found a greater loss of sodium from the extracellular fluid than could be accounted for by external losses(10,11). They suggested that the sodium was sequestered in the bone. However, White, *et al.*(6) were unable to confirm these findings in adrenalectomized rats.

The present investigation was carried out in an attempt to clarify the role of the adrenal gland in bone electrolyte metabolism.

Materials and methods. Female Sprague Dawley rats were used for all experiments. At time of sacrifice the animals were given pentobarbital 6 mg/100 g I.P. Blood was drawn from the right ventricle and the serum separated. Sera were pooled to obtain samples for electrolyte determinations.

The femurs with cartilage removed but with marrow present were weighed in the fresh state and again after drying for at least 48 hours at 105°C. The dry bones from 3 or 4 rats were ground together in a mortar and 2 samples of the powder were taken. These were prepared for sodium and potassium determinations by the method of Bergstrom and Wallace(2). Our technic yielded 95% of the Na and 90% of the K present in a mixture of various salts approximately the composition of bone.

Sodium and potassium concentration in bone and serum samples was determined by flame photometry, using lithium as an internal standard.

Adrenalectomies were done through bilateral subcostal incisions and the rats were maintained on Purina Laboratory Chow and tap water until the fourth postoperative day when they were sacrificed. Half the adrenalectomized rats were controlled by simultaneous measurements on unoperated rats selected at random from the same group. The other half were compared to controls of similar weight but not measured simultaneously. There was no difference between the subgroups and they are grouped together for statistical analysis.

A number of different steroid-administration experiments were done with simultaneous controls selected from the same group. All these animals were maintained on Purina Chow. Groups A and B were both given saline instead of tap water for drinking during the experiment. Group C received tap water. Each animal in subgroup A steroid-treated received 5 mg desoxycorticosterone trimethylacetate i.m. at the start plus 1.0 mg desoxycorticosterone acetate (DOCA) daily for 4

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† This investigation was carried out during the tenure of a Post-doctoral Fellowship from Nat. Heart Inst., U.S.P.H.S. Present address U. S. Naval Station, Navy 117, Fleet Post Office, New York. The opinions and statements contained herein are the private ones of the author and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

TABLE I. Bone and Serum Electrolytes of Normal and Adrenalectomized Rats.

			Mean serum electrolytes (meq/l)			Mean bone electrolytes (meq/kg fresh bone)			
Group	No. of rats	Mean wt at sacrifice, g	No. of measurements	Na	K	No. of measurements	Na	K	Bone water conc., %
Control	22	132	11	139	5.1	12	132	49	40
S.E.		1.3		1.0	.7		2.8	2.3	
Adrenx	20	119	11	132	6.2	10	92	45	40
S.E.		2.5		1.4	1.7		8.7	1.9	
p		.01		.1	.2		<.01	<.2, >.1	

days, and were sacrificed 18 hours after the last dose. Each in subgroup B steroid-treated received 1.0 mg DOCA daily for 4 days, then was sacrificed. Subgroup C steroid-treated animals each received one injection of 12.5 mg desoxycorticosterone trimethylacetate and were sacrificed 2 weeks after injection. (Serum electrolytes on different animals in this laboratory on this regimen show the expected fall in potassium concentration with no change in sodium.)

All measurements for bone electrolytes are in units of mEq per kg fresh whole femur. No attempt was made to calculate "chloride space sodium" or "free sodium" since our interest was in the net sodium or potassium balance of whole bone.

Results. Our measurements of adrenalectomized compared to control rats are summarized in Table I. Weights given are those at time of sacrifice and reflect dehydration of the adrenalectomized animals. Bone sodium concentration decreased significantly in the adrenalectomized rats. The potassium appeared to decrease slightly but the change was not statistically significant for the number of measurements.

The results of steroid treatment are reported in Table II. Neither bone sodium nor bone potassium changed significantly, although in each experiment, average potassium values for steroid-treated rats were slightly lower than the controls.

Discussion. This investigation confirms the results of others showing that adrenalectomy causes a decrease in bone sodium(6,7, 8,9). It does not support Stern, Cole, Bass, and Overman's(10) conclusions of no change. These workers, however, actually found a fall in mean bone sodium levels in their dogs

TABLE II. Bone Electrolytes of Normal and Steroid Treated Rats.

Group	No. rats	Mean wt, g	No. measurements	Na	K	Bone water conc., %
				meq/kg		
Control A	7	149	4	145	45	36
S.E.		3.3		3.6	2.8	
Steroid A	7	146	4	156	43	35
S.E.		4.5		9.8	2.1	
p				.3	.9	
Control B	8	171	4	155	42	36
S.E.		6.1		9.5	2.8	
Steroid B	8	168	4	150	38	36
S.E.		5.6		3.0	.6	
p				.6	<.3, >.2	
Control C	11	172	6	—	40	37
S.E.		2.9		—	2.0	
Steroid C	12	180	6	—	38	37
S.E.		3.3		—	1.4	
p					<.2, >.1	

from 246 to 229 mM/kg fresh midfemur. Because of the large variation between measurements and small number of measurements made, this fall was not statistically significant.

Munro, Satoskar, and Wilson(8) did not demonstrate a fall in bone sodium in adrenalectomized rats maintained on 0.9% saline when compared to normal rats on a regular diet and tap water. They did find a fall in adrenalectomized rats on a rice diet and distilled water, but had already shown(3) that a rice diet and distilled water can lower bone sodium in normal rats. They did not report results of adrenalectomized rats on a standard diet and water or normal rats on saline. Nichols and Nichols(12) have shown that salt alone, in large quantities, could actually raise bone sodium.

The effects of adrenal steroid administration on bone electrolyte composition have not been thoroughly studied. Dosekun(9) found that cortisone treatment prevented the fall of bone sodium concentration in adrenalectomized animals. Borle, *et al.*(7), demonstrated that prednisolone did not change bone Na or K concentrations in intact rats. Woodbury(13) and Green *et al.*(14) using a radioactive sodium technic found that mineraloactive corticoids did not elevate bone sodium concentration above normal. Using a direct measurement technic and various mineraloactive corticoid regimens, we were unable to produce a significant change in bone Na or K concentrations in intact rats.

Summary. The effects of adrenocortical hormones on bone electrolyte metabolism have been investigated by 2 series of experiments. In the first, femur sodium and potassium concentrations were compared in normal and adrenalectomized rats. Sodium concentration decreased a significant amount. In the second series, normal rat femurs were compared to femurs from rats given varying amounts of NaCl in the diet and various mineraloactive steroid drugs. In each of these experiments sodium concentration was unchanged and potassium fell an insignificant amount.

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1. Harrison, H. E., *J. Biol. Chem.*, 1937, v120, 457.
2. Bergstrom, W. H., Wallace, W. M., *J. Clin. Invest.*, 1954, v33, 867.
3. Munro, D. S., Satoskar, R. S., Wilson, G. M., *J. Physiol.*, 1957, v139, 474.
4. White, H. L., Rolf, D., *Am. J. Physiol.*, 1955, v180, 287.
5. Levitt, M. F., Turner, L. B., Sweet, A. Y., Pandiri, D., *J. Clin. Invest.*, 1956, v35, 98.
6. Rolf, D., Armstrong, C. M., Steiger, B. W., Audia, M., White, H. L., *Am. J. Physiol.*, 1959, v196, 1109.
7. Borle, A. B., Nichols, G., Jr., Karnovsky, M. J., *Endocrinology*, 1960, v66, 508.
8. Munro, D. S., Satoskar, R. S., Wilson, G. M., *J. Physiol.*, 1958, v142, 439.
9. Dosekun, F. O., *ibid.*, 1959, v147, 115.
10. Stern, T. N., Cole, V. V., Bass, A. C., Overman, R. R., *Am. J. Physiol.*, 1951, v164, 437.
11. Flanagan, J. B., Davis, A. K., Overman, R. R., *ibid.*, 1950, v160, 89.
12. Nichols, N., Nichols, G., Jr., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 835.
13. Woodbury, D. M., *Am. J. Physiol.*, 1953, v174, 1.
14. Green, D. M., Raynolds, T. B., Girerd, R. J., *ibid.*, 1955, v181, 105.

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Properties of a Transmissible Agent Capable of Inducing Marked DNA Degradation and Thymine Catabolism in a Human Cell.* (26558)

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Following the establishment of a strain of epithelial-like cells from a biopsied specimen of human liver in 1954(1), attempts were made to propagate the infectious hepatitis virus in this cell. A transmissible cytopathic agent appeared during one of those attempts. Although the origin and identity of this agent has not been ascertained, its many unusual features, its history and general properties are described in this report. The unique property of inducing marked DNA degradation and thymine catabolism in the "liver" cells is described in an accompanying report(2).

Materials and methods. Cell cultures. Monolayer roller cultures nourished in a nutrient medium consisting of 5% horse serum, preheated at 56°C for 30 minutes, in Eagle's basal medium have been used exclusively since 1956(3). Prior to 1956, a nutrient medium consisting of 20% heated horse serum in Scherer's MS(4) was used. Chick plasma, chick embryo extract and several human sera had also been used in establishment of this cell line. The "liver" cell culture(1) was found contaminated with a pleuropneumonia-like organism in 1956. Since 1957, following prolonged treatment with tetracycline, the culture has been apparently free of any such contaminants when tested on Edward's serum-agar(5), nutrient agar containing 15% horse blood and thioglycollate broth.

History of the agent. In December 1954, 4 "liver" cultures were inoculated with blood freshly drawn from 4 hepatitis patients. The blood specimen was collected on the 1st or 2nd day of jaundice during an outbreak of infectious hepatitis in a state institution for mentally defective children. About 30 days later, a blind passage was made by transferring cells and media from these 4 cultures into one single "liver" culture. In 20 more days, rapidly progressing cytopathic change appeared. This cytopathic change may be readily transmitted to other liver cultures by transferring some degenerated cells. More than 100 serial passages have been made. The data to be described are based on studies made in 1959 and 1960 on this agent which has undergone numerous passages in the "liver" culture since 1954. Unless specified otherwise, the "liver" culture was used exclusively in experiments described here.

Results. Cytopathology. Following the transfer of 0.2 ml of medium and degenerated cells from a completely degenerated culture (previously infected with the agent) to a healthy culture, degeneration usually appeared in 1 or 2 days and progressed rapidly to completion within 2 or 3 days. The degeneration is characterized by cytoplasmic granularity followed by cell rounding and shrinkage. Infected shrunken cells apparently remained intact morphologically for at least 4 weeks when kept at 36°C. Stained preparation of partially degenerated cultures showed characteristic nuclear lesion consisting of basophilic globular bodies frequently situated next to the nuclear membrane (Fig. 1). These basophilic globules are Feulgen positive, and are regularly seen in infected "liver", infected mouse fibroblasts (strain L) and in those successfully infected primary human amniotic cells.

Due to factors yet to be identified, the infecting process may at times show 3 other features: (1) marked prolongation of the incubation period; (2) establishment of a "chronic" infection, and, (3) reappearance of colonies of healthy cells in apparently completely degenerated cultures. In 2, the culture appeared partially degenerated but continued to shed infectious material for at least 2 months and was capable of converting thymidine-2-C^{14} to $C^{14}O_2$. In 3, the colonies which re-emerged were found destroyed following reintroduction of fresh agent.

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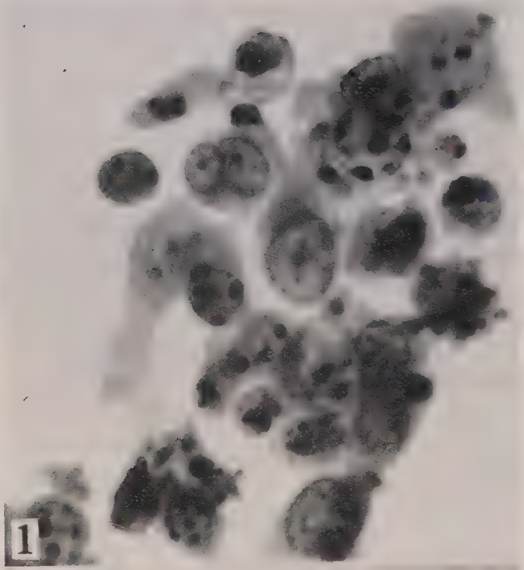

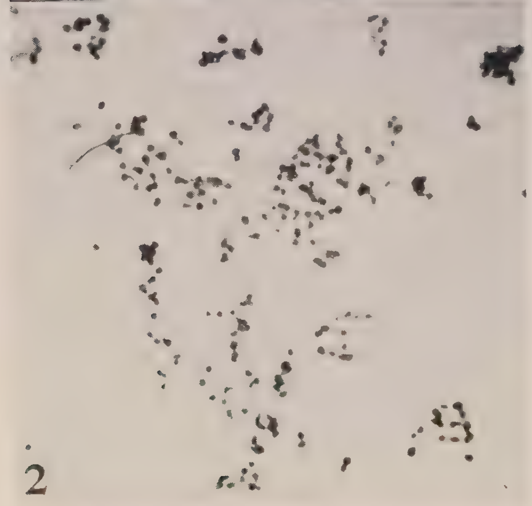

FIG. 1. Basophilic globular bodies in nuclei of infected "liver" cells.

FIG. 2. Infective portion of a degenerated "liver" culture. Note relative uniformity in size and shape of these "shrunken cells." Diameter is slightly larger than that of a human erythrocyte.

Some physico-chemical properties. The infectivity of this agent was cell associated; the fluid phase of infected culture did not contain demonstrable infectious material. The infectious component was completely sedimented by centrifugation at 2000 rpm for 10 minutes in a horizontal centrifuge (International) and was retained by a membrane filter (Millipore) with pore size of 5 μ. The material retained by this filter was infectious and consisted of many shrunken cells relatively uniform in size (about 8-12 μ in diameter) and shape (Fig. 2). The infectivity of an infected culture was destroyed by 1 cycle of freeze-thawing (occasional exceptions were encountered) but was partially retained for at least 16 weeks at 2-4°C, and 8 weeks at 36°C. Infected mouse fibroblasts cultures also retained their infectivity after heating at 56°C for 1 hour. Attempts to release infectious particles by homogenization (with a Potter homogenizer) resulted in marked or complete loss of infectivity. Exposure to .85% sodium chloride solution buffered at pH of 5 and 8 with M/15 phosphate for 24 hours at 25°C did not detectably affect its infectivity or cause the release of infectious particles. In such studies, failure to produce characteristic cytopathic changes in the "liver" cell 14 days after infection is arbitrarily considered negative for infectivity.

Susceptibility of different types of cells in vitro. Using an inoculum capable of causing complete degeneration of the "liver" cells in 3 or 4 days, cytopathic changes leading to complete destruction were produced in the following cell lines: conjunctival, HeLa, KB, FL and appendix derived from human tissues, and mouse fibroblast, strain L, and sarcoma 180 II of murine origin. Interestingly, when infected with a similar dose, primary explants of postnatal human amniotic cells (more than 10 trials) and renal cells (2 trials) or simian renal cells (3 trials) failed to show distinct cytopathic changes. With a larger infective dose, the primary amnion culture may also show characteristic degenerative changes.

Immunological study. Infected "liver" cultures, showing partial or complete degeneration, neither hemagglutinate nor hemadsorb human group "O", sheep or chick erythrocytes. Using as antigen infected mouse fibroblast L or "liver" cells in a serum free medium, negative complement fixation reactions were obtained with the following serums: (1) paired acute and convalescent serums from 5 infectious hepatitis patients (fresh blood specimens used in the original isolation were obtained from 3 of these 5 patients): (2) two convalescent varicella serums of known high antibody titers against varicella antigen; (3) two convalescent rubella serums; (4) one adenovirus convalescent serum of high antibody titer; (5) two convalescent serums from patients with undiagnosed fever, and (6) a pool of serum from 10 healthy adults. Pooled horse serum (from 6 horses) and mouse serum (20 white Swiss mice) also gave negative CF reaction. Indirect fluorescent antibody study revealed no demonstrable fixation of antibody on the nuclear lesions of infected human "liver" or mouse fibroblasts; serums of category 1 were used. Although all the aforementioned serums also failed to neutralize this agent, these neutralization tests had to be considered inadequate since the specifically involved shrunken cells were used as the infectious material.

Other properties. This agent does not produce any visible illness in baby or adult white Swiss mice by intracerebral or intraperitoneal routes. Chick embryos survive infection by the yolk sac route, and rabbits, by intravenous or intraperitoneal routes. When infectious material was plated on blood agar or Edward's serum-agar medium and incubated in an atmosphere of 5% CO_2 in air at 36°C, no visible growth could be detected after 7 days. Similarly, no detectable growth appears in thioglycollate broth. Addition of tetracycline at 10 units per ml medium does not affect the progression of the cytopathic changes.

Failure to isolate similar agent. Several attempts to reisolate a similar agent from the 4 original specimens kept frozen at below –60°C were unsuccessful. Similarly, several other frozen acute hepatitis bloods and pooled stool specimens from 2 epidemics of infectious hepatitis failed to yield any transmissible cytopathic agent. It must be emphasized, however, that these attempts were made with frozen material and that freeze-thawing readily inactivates this agent. Isolation study using fresh acute hepatitis bloods is currently being made.

Discussion. Data obtained to date fail to establish the origin and identity of this agent. Serological findings obtained thus far neither establish nor exclude its immunological relationship with the infectious hepatitis virus. Various methods for release of this agent from the specifically involved shrunken cells are currently being investigated so that neutralization tests may be more adequately performed. Irrespective of its origin and identity, many of its characteristics are unusual for a virus infectious for human cells. These unusual features are: appearance of large basophilic Feulgen positive globular bodies in the nucleus; greater susceptibility of established cell lines as compared to the primary amnion or kidney; complete association of infectivity with the specifically involved shrunken cells; remarkable stability of infectivity of the shrunken cells and apparent rapid loss following procedures which interrupt its structural integrity, such as freeze-thawing and homogenizing, and induction of host DNA degradation and thymine catabolism, described in the accompanying report (2). Certain resemblance to the varicella-zoster group of viruses may be mentioned. The association of the infectivity with the degenerating cell and loss of infectivity following freeze-thawing and homogenizing have been reported for tissue culture propagated varicella-zoster viruses(6). Absence of eosinophilic intranuclear inclusions, failure to demonstrate viral proteins specific for varicella by the complement-fixation tests and the reaction of the cultured cells, to name only a few factors clearly establish the non-identity of the varicella-zoster group of viruses and this agent.

A logical hypothesis for the great stability of infectivity of the specifically involved shrunken cells and almost instantaneous loss of infectivity following mechanical disintegration of these cells is as follows: (1) the agent is enzyme-sensitive, and some of the cells are held at locale not accessible to the enzymes, and (2) the loss of structural integrity of the shrunken cells results in prompt enzymatic inactivation of the agent.

The rapid destructive action of this agent on the rapidly growing established human cells in contrast to its benign effect on the relatively dormant primary amniotic cells suggests a remote possibility of an oncolytic agent. Its ability to destroy the rapidly growing mouse fibroblasts, strain L, and sarcoma 180 II cells in contrast to its lack of pathogenicity for the mouse, and its capability of inducing DNA depolymerization and thymidine catabolism in the susceptible cell are also in keeping with the requirements of an effective oncolytic agent. This possibility is being explored.

Summary. The appearance of a transmissible cytopathic agent during the first blind passage of pooled human "liver" cultures previously infected with 4 acute infectious hepatitis bloods is described. The infectivity of this agent in the "liver" culture was cell-associated. The infective degenerated "liver" cells retained their infectivity for at least 16 weeks at 4°C and 8 weeks at 36°C. Heating for 1 hour at 56°C failed to destroy completely the infectivity of infected mouse fibroblasts. One cycle of freeze-thawing or homogenizing, however, abolished the infectivity with occasional exceptions. Characteristic cytopathology consisted of cytoplasmic granularity, cell rounding followed by diminution of cell volume leading to morphologic picture of shrunken cells. During an intermediate stage of degeneration, characteristic intranuclear basophilic globules were seen regularly; the globules were Feulgen positive. The established cell line was more susceptible to the destructive action of this agent than primary cells. Preliminary immunological study failed to establish the identity of this agent.

1. Chang, R. S., Proc. Soc. Exp. Biol. and Med., 1954, v87, 440.

2. ———, Proc. Soc. Exp. Biol. and Med., 1961, v107, 138.

3. ———, Proc. Soc. Exp. Biol. and Med., 1957, v96, 336.

4. Scherer, W. F., Syverton, J. T., Gey, G. O., *J. Exp. Med.*, 1953, v12, 264.

5. Edward, D. G., *J. Gen. Microbiol.*, 1954, v10, 27.

6. Weller, T. H., Witton, H. M., Bell, E. J., *J. Exp. Med.*, 1958, v108, 843.

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Appearance of Marked DNA-Degrading and Thymine Catabolic Activities in a Human Cell Infected with a Transmissible Agent.* (26559)

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The preceding paper described the history and general properties of this unidentified transmissible cytopathic agent(1). The appearance of DNA-degrading and thymine catabolic activities in infected "liver" cultures is described in this report.

Materials and method. Cell cultures. Roller cultures were used exclusively. The "liver" cells(2) were grown directly on glass in monolayer. Nutrient medium consisted of 5% horse serum, preheated at 56°C for 30 minutes, in Eagle's basal medium fortified with double strength amino acids and the non-essential amino acids at 0.2 mM(3). Certain modifications of this basal medium were also made(4).

C^{14} labelled substrates. Thymidine-2-C^{14}, uridine-2-C^{14}, and uracil-2-C^{14} with specific activity of 6.29, 0.65 and 1.1 mC per mMole respectively, purchased from New England Nuclear Corp., were used at final concentrations of 0.5 μC per ml medium.

C^{14} labelled culture. "Liver" cultures grown in media containing either thymidine-2-C^{14} or uridine-2-C^{14} for 48 to 72 hours, were rinsed 3 times with 2 ml of regular medium, without C^{14} substrate, then allowed to grow for another 24 to 48 hours in the regular media.

Fractionation of C^{14} labelled cell culture. CO_2 was first absorbed with an alkaline trap suspended in the gaseous phase of the culture

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TABLE I. C^{14} Activity in Various Fractions of Uninfected Thymidine-2-C^{14} or Uridine-2-C^{14} Labelled "Liver" Cells.

		C^{14} activity (cpm) in fractions					
Cells labelled with	Exp.*	Acid soluble	Lipids	RNA	DNA	Protein	CO_2
Thymidine-2-C^{14}	1	3	0	21	885	225	0
	2	66	0	69	2104	738	0
Uridine-2-C^{14}	3	224	4	3537	1025	225	0
	4	1552	0	1134	540	108	2

* In Exp. 1, cells were grown in thymidine-2-C^{14} containing medium for 48 hr; cells were rinsed twice and further propagated in medium without thymidine for 3 more days.
A total of 765,000 cells was fractionated.
In Exp. 2, 2 × 10^6 cells were fractionated after 48 hr in thymidine-2-C^{14} containing medium.
Exp. 3 is similar to 1 except uridine-2-C^{14} was used and 1.6 × 10^6 cells fractionated.
Exp. 4 is similar to 2 except uridine-2-C^{14} was used.

(5). The whole culture, medium and cells, was then fractionated into fractions 1 (acid soluble), 2 (lipids), 3 (RNA), 4 (DNA), and 5 (proteins) by the method of Schmidt-Tannhauser-Schneider(6). Preliminary study showed that C^{14} activity of the labelled culture resided almost entirely in the nucleic acids and protein. Subsequently, the culture was merely fractionated into cold acid soluble and insoluble fractions, the latter containing the nucleic acids. In actual experiments, the cells (those adhered on glass) and the medium (including detached degenerating cells and fragment) were fractionated separately.

Infectious inocula. Completely degenerated cultures of "liver" cells infected with the agent were centrifuged at 2000 rpm for 10 minutes in a horizontal centrifuge. The sediment, which contained the infectivity, was washed 3 times with 0.25 M sucrose containing 0.003 M $CaCl_2$, and was resuspended in sufficient sucrose solution so that 0.2 ml would contain sufficient infectivity to cause complete degeneration of a liver culture in 2 or 3 days. Other viral agents used for comparative study were vaccinia, adeno 3, herpes simplex, polio 1 and Coxsackie B1. The vaccinia virus was prepared from the chorio-allantoic membrane of the chick embryo, the other viruses from cultures of HeLa cells. The dosage of these viruses was also adjusted so that 0.2 ml contained sufficient virus to cause complete degeneration of a "liver" culture within 2 or 3 days.

DNA or RNA degradation study. Two of a replicate set of C^{14} labelled cultures were first fractionated (considered zero hour). The remainder were then infected by the various agents. A culture from each set was fractionated on the 24th, 48th, and 72nd hour of experiment. Regular media without C^{14} substrate were used.

Measurement of C^{14} activity. Thin specimens containing 0.5 of each fraction were prepared. Their C^{14} activity was monitored with a gas flow Geiger-Mueller tube of about 15% efficiency. Total time required to register 1000 counts was determined for each sample. The results were expressed as counts per minute above background per culture. Net values less than 20% of the background count (5 or less) were arbitrarily considered insignificant. No attempt was made to remove the trichloracetic acid in the acid soluble fraction. This amount of trichloracetic acid reduced the C^{14} activity of a sample by approximately one-half.

Results. Distribution of radioactivity in various fractions of "liver" cells grown in thymidine-2-C^{14} or uridine-2-C^{14} containing medium. Several experiments were performed to determine if the "liver" cell, like most other cells, utilizes thymidine solely for DNA synthesis and uridine for RNA and DNA syntheses. Results are presented in Table I; C^{14} activity does reside mostly in the RNA and DNA fractions. Of interest is the failure to detect radioactivity in the CO_2 evolved by this cell, suggesting that the mechanism for reductive catabolism of uracil or thymine (7) is inhibited or absent. The C^{14} activity of the protein fraction is believed due to incomplete separation of DNA from protein; that in the DNA fraction of uridine-2-C^{14}

TABLE II. Radioactivity in CO_2, Acid Soluble and Insoluble Fractions of Thymidine-2-C^{14} Labelled "Liver" Cells Treated with Various Viral and Physical Agents.

		Radioactivity* in fractions							
Agent	Day		CO_2	Acid soluble	Acid insoluble		CO_2	Acid soluble	Acid insoluble
None	0	(0) †	0	5	1528	(0) †	2	10	4694
	1	(0)	0	4	1542	(0)	0	14	4756
	2	(0)	0	10	1602				
	3	(0)	0	4	1376	(0)	2	60	3928
The agent	1	(+)	20	23	1398	(++)	516	368	1878
	2	(++)	108	120	698				
	3	(+++)	274	232	210	(+++)	822	434	216
Adeno 3‡	1	(+)	0	8	1658	(+)	2	12	4088
	2	(++)	0	14	1468				
	3	(+++)	1	62	1540	(++)	2	34	3012
Freeze-thawing	1	(+++)	0	6	1328	(+++)	0	22	3490
	2	(+++)	0	4	1262				
	3	(+++)	0	14	1538	(+++)	4	44	2774

* cpm above background per culture.

† 0, +, ++ and +++ signified respectively no, early, advanced and complete degeneration.

‡ Vaccinia, herpes simplex, polio 1 and Coxsackie B1 gave similar results.

labelled cells may be readily explained by the well known conversion of uridylate to thymidylate; while the low activity in the RNA fraction of thymidine-2-C^{14} labelled cells is probably due to a partial hydrolysis of DNA during separation of these 2 nucleic acids(8). It seems reasonable to conclude that, for our purpose, most if not all thymidine is utilized in DNA synthesis, and most or all uridine, in RNA, DNA and acid soluble nucleotides syntheses.

DNA degradation and thymine catabolism. Following infection of thymidine-2-C^{14} labelled cells with the agent, C^{14} activity of the CO_2 and acid soluble fraction increases while that of the acid insoluble fraction decreases in thymidine-2-C^{14} labelled cultures. No such changes have been observed for uninfected healthy cultures, cultures killed by freeze-thawing, cultures nourished in a deficient medium consisting of balanced salt solution minus glucose, and cultures infected by vaccinia, adeno 3, herpes simplex, Coxsackie B1 or polio virus. Results of 2 such experiments are shown in Table II. The apparent loss of total radioactivity of cultures infected by the agent may be due to our inability to collect all the CO_2 evolved and the interference of C^{14} measurement by trichloracetic acid present in the acid soluble fractions.

To determine if the absence of thymine-2-C^{14} to $C^{14}O_2$ conversion in cultures other than those infected by this DNA-degrading agent may be due to an insufficient degradation of DNA, the experiments were repeated using unlabelled culture in thymidine-2-C^{14}, uridine-2-C^{14} or uracil-2-C^{14} containing medium. Again only the CO_2 evolved from cultures infected with the agent shows significant radioactivity.

RNA degradation. When these experiments were repeated using uridine-2-C^{14} labelled cells, vastly different results were obtained. Increases of C^{14} activity in the acid soluble fractions were found under all experimental conditions leading to cell death. The liberation of significant quantity of $C^{14}O_2$ was, however, restricted to cultures infected with the agent. Table III shows the results of such an experiment. Since RNAse has been demonstrated in horse serum, it is possible that the serum RNAse may be responsible for the RNA degradation following cell death. To rule out this possibility, the experiment was repeated using the "liver" cell adapted to grow in a chemically defined medium by Holmes(9). Again, results similar to those presented in Table III were obtained.

Discussion. The appearance of DNA degradative activities in the human "liver" cells following infection with this unidentifiable transmissible cytopathic agent seems well established. These degradative activities consist of conversion of acid insoluble polynu-

TABLE III. Radioactivity in CO_2, Acid Soluble and Insoluble Fractions of Uridine-2-C^{14} Labelled "Liver" Cells Treated with Various Agents.

			C^{14} activity, cpm/culture, in		
Agent	Day	Degeneration*	CO_2	Acid soluble	Acid insoluble
None	0	0	0	60	3522
	1	0	0	72	2654
	3	0	2	256	1800
The agent	1	++	78	138	2094
	3	+++	270	282	512
Adeno 3	1	+	2	74	2868
	3	++	0	172	1825
Freeze-thawing	1	+++	2	322	2214
	3	+++	0	540	1524
Vaccinia†	3	+++	4	742	1232

* 0, +, ++ and +++ represented no, early, advanced and complete degeneration respectively.

† Polio 1, Coxsackie B1, and herpes simplex gave similar results.

cleotides to acid soluble components and the cleavage of thymine with CO_2 formation. It is not known whether such active degradative activities are secondary to cell death although they must be associated with cell death. The real significance is that such degradative activities are seen only in cells killed by this transmissible agent and not in those killed by other agents (*e.g.*, 3 DNA viruses, 2 RNA viruses, freeze-thawing and 1 form of multiple nutrient depletion). The possible presence of low DNAse activity in cultures killed by these various other agents is suggested by the small but fairly consistent increase in radioactivity of the acid soluble fractions.

Study on induced RNA degradation was obscured by the finding of significant conversion of acid insoluble to acid soluble radioactive compounds in uridine-2-C^{14} labelled cultures which had degenerated under a variety of experimental conditions. The RNA degradation in uninfected healthy culture may be best explained by the well known observation that, in most monolayer culture, a varying portion of the total cell population is constantly undergoing degenerative changes.

To our knowledge, such extensive degradation of host DNA into acid soluble nucleotides following infection by any transmissible agents has been reported for a few bacterial viruses(10). Induction of reductive catabolism of thymine and uracil in a host cell by a transmissible agent has hitherto not been described. The exact mechanism of these induced changes and the nature of this transmissible agent are currently being investigated.

Summary. Following infection by a transmissible cytopathic agent and with the appearance of degeneration, the following were regularly observed for thymidine-2-C^{14} labelled human "liver" cultures: 1. progressive increase in quantity of $C^{14}O_2$ liberated, 2. progressive increase in radioactivity of the acid soluble fractions, and 3. progressive decrease in radioactivity of the acid insoluble fractions. Similar changes had not been demonstrated in uninfected culture, culture killed by freezing or by nutrient depleting, and cultures infected with vaccinia, adeno 3, herpes simplex, polio 1, or Coxsackie B1 viruses. Significant conversion of acid insoluble to acid soluble radioactive compounds was repeatedly demonstrated in uridine-2-C^{14} labelled "liver" cultures under all the described experimental conditions leading to cell degeneration. Formation of $C^{14}O_2$ from uracil-2-C^{14} or uridine-2-C^{14} was, however, restricted to cultures treated with this DNA-degrading agent.

The authors are grateful to Dr. Holmes, Alfred I. du Pont Institute for making available to us a strain of "liver" cells adapted to grow in Holmes' synthetic medium, and for a generous supply of his chemically defined medium.

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1. Chang, R. S., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 135.

2. ———, PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 440.

3. Eagle, H., *Science,* 1955, v122, 501.

4. Chang, R. S., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 336.

5. Geyer, R. P., Chang, R. S., *Arch. Biophys. and Biochem.,* 1958, v73, 500.

6. Glick, D., *Methods of Biochemical Analysis,* 1957, v1, 290, Interscience Publishers, New York.

7. Davidson, J. N., *The Biochemistry of the Nucleic Acids.,* 1959, Metheun and Co.

8. Drasher, M. L., *Science,* 1953, v118, 181.

9. Holmes, R., *J. Biophys. Biochem. Cytol.,* 1959, v6, 535.

10. Kozloff, L. M., *Cold Spring Harbor Symposia Quant. Biol.,* 1953, v18, 207.

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Plaque Inhibition Test for Detection of Specific Inhibitors of DNA Containing Viruses. (26560)

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Recently it has been reported that 5-bromo- and 5-fluoro-2′-deoxyuridine (BDU and FDU) not only interfered with normal deoxyribonucleic acid (DNA) biosynthesis in animal and bacterial cells but also specifically inhibited viral DNA synthesis(1-3). Using the agar-diffusion plaque-inhibition test(4), it was discovered that still another member of this series, 5-iodo-2′-deoxyuridine (IDU), was a potent inhibitor of plaque formation by vaccinia and herpes simplex viruses which are considered to contain DNA. Plaque formation of ribonucleic acid (RNA) containing West Nile (WN) and Newcastle disease (ND) viruses was not inhibited. The usefulness of the plaque inhibition test for detection of such inhibitors was examined in the present study. In addition to testing the halogen derivatives of deoxyuridine a variety of other related compounds was examined, some of which might be expected to have an influence on nucleic acid biosynthesis. Data are included to show that the agar-diffusion test also is useful for detecting thymidine reversal of IDU and BDU plaque inhibition.

Materials and methods. Preparation of agar overlay, chick embryo cell cultures in Pyrex baking dishes, infection of these cultures with vaccinia, herpes, WN and ND viruses, the strains used, subsequent staining of the cells with iodonitrotetrazolium[†] chloride to observe plaques and detection of zones of plaque inhibition previously have been described(4). Both growth (lactalbumin-calf serum) and agar overlay (lactalbumin-yeast extract) media were slightly modified by replacing the bicarbonate buffer with Tris-HCl buffer.[‡] In addition, 90 mm plastic Petri dishes (tissue culture type, Falcon Plastics) also were used with proportional amounts of cells, media, etc. as previously described for the baking dish cultures. *Test compounds* were stored at 4°C, and at the time of the test a small amount of each was deposited directly on the surface of a freshly infected agar overlay culture. It was estimated that the quantity tested by this technic was no less than 200 μg. Generally, 200 μg can be considered a high level of material in this type of test. When plaque inhibition was observed, the compound was retested at known concentrations in a volatile solvent, usually dimethylformamide. One-quarter inch filter paper discs were impregnated with 0.02 ml of solution and then air dried. The discs then were arranged on the surface of freshly infected agar overlay cultures.

Results and discussion. The data show (Table I) that as little as 1-2 μg of IDU or BDU inhibited plaque formation by vaccinia and herpes simplex viruses while 200 μg did not influence WN and ND viruses. Plaque inhibition was not observed with any of the other compounds including 3 samples of aminopterin. This compound has been reported, however, as an inhibitor of normal DNA biosynthesis and a specific inhibitor of DNA containing polyoma virus in mouse embryo cell cultures(1). There are numerous possible reasons for this apparent discrepancy including differences in viruses, host cells, media, and technic. BDU, however, was active in both systems. FDU, on the other hand, has been reported as a specific

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[†] In the original reference(4) this compound was spelled incorrectly as indonitrotetrazolium chloride. The correct name of the compound is 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride or INT.

[‡] To a 100 ml 2M solution of tris-hydroxymethylaminomethane was added 2M HCl to bring the pH to 7.6, and then the volume was brought to 200 ml. This stock solution was added at the rate of 1.8 ml to each 100 ml of media. A small amount of bicarbonate also was present (1 ml per 100 ml of media of a 7.5% $NaHCO_3$ solution).

TABLE I. Effect of Various Compounds on Virus Plaque Development.

Compound and source	Concentration	Plaque-free zone diameter (mm)			
		Vaccinia	Herpes simplex	West Nile	Newcastle
5-Iodo-2′-deoxyuridine (NBC)	200 μg/disc	65	60	0, 0	0, 0
Idem	20 ”	55, 53, 47	50		
”	10 ”	28	30		
”	5 ”	26			
”	2 ”	22			
”	1.25 ”	18	20		
5-Bromo-2′-deoxyuridine (NSC 38297)	200 ”	60–70	45, 50	0	0, 0
Idem	20 ”	58	35, 35	0	0, 0
”	10 ”		30		
”	5 ”		25		
”	2.5 ”		trace		
5-Fluoro-2′-deoxyuridine* (NSC 27640)	>200 μg	0, 0, 0, 0	0, 0, 0	0	0
Aminopterin (NSC 739)	>200 ”	0, 0, 0	0, 0, 0	0	0
” (Lederle)	>200 ”	0	0	0	0
” (CCBR)	>200 ”	0	0	0	0
Deoxyuridine (CCBR)	>200 ”	0, 0, 0	0, 0, 0	0	0
5-Bromouridine (NBC)	>200 ”	0, 0	0, 0	0, 0	0
Uridine (NBC)	>200 ”	0	0, 0	0	0, 0
5-Hydroxyuridine (NBC)	>200 ”	0	0	0	0
6-Azauridine (NBC)	>200 ”	0	0	0	0
Uridine-5-phosphate, Na salt (NBC)	>200 ”	0	0	0	0, 0
Iso-propylidene uridine (NBC)	>200 ”	0	0	0	0
Thymidine (CCBR)	>200 ”	0, 0, 0	0, 0, 0	0	0
5-Iodouracil (CCBR)	>200 ”	0	0, 0	0	0, 0
5-Bromouracil (CCBR)	>200 ”	0, 0	0, 0	0	0
6-Azauracil (NSC 3425)	>200 ”	0	0	0	0
5-Fluorouracil (NSC 19893)	20 μg/disc	0, 0	0, 0	0	0
5-Bromo-1-methyl-hydrouracil (NSC 42058)	20 ”	0	0	0	0
6-Propylthiouracil (CCBR)	>200 μg	0	0	0	0
Thymine (CCBR)	>200 ”	0, 0	0, 0	0, 0, 0	0
Deoxyadenosine (CCBR)	>200 ”	0	0, 0	0	0
Adenosine (CCBR)	>200 ”	0	0	0	0
Adenine (CCBR)	>200 ”	0	0	0	0
Orotic acid (CCBR)	>200 ”	0, 0	0	0	0, 0

NBC = Nutritional Biochemicals Corp. NSC = Cancer Chemotherapy Nat. Service Center. CCBR = California Corp. for Biochemical Research.

* A new sample of this compound (Batch No. 30—Hoffmann-LaRoche) was recently received and tested at 200, 20 and 2 μg per test disc against vaccinia and herpes simplex viruses. Plaque suppression was again not observed.

DNA inhibitor for both vaccinia and herpes simplex viruses(2,3). In this case, again, there are differences in technic, host cells and media. It is worth noting that FDU has been reported as an inhibitor of thymidilic acid biosynthesis while IDU inhibits the incorporation of thymidine into DNA and is itself incorporated, resulting in so-called fraudulent DNA(5). Aminopterin also has been reported to influence DNA synthesis by interfering with thymidilic acid production(1). Perhaps interference with thymidilic acid biosynthesis is not an adequate mode of action for suppressing plaque formation in the system used here. Additional samples of FDU are being obtained so that the reason for the apparent discrepancy in the antiviral activity of this compound can be studied.§

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§ The question has been raised as to whether halogenated deoxyuridines specifically inactivated DNA viruses rather than influenced their synthesis. The methods used by previously cited investigators (1,2) seem to have eliminated this possibility. Furthermore, studies in this laboratory with numerous virus inactivating substances have shown that the plaque-inhibition test is relatively insensitive to such materials, probably because they are applied sometime after the cells are infected.

TABLE II. Reversal by Thymidine of Plaque Inhibition Produced by 5-Halogenated Deoxyuridines.

	Plaque inhibition zone radii (mm)*			
	Vaccinia		Herpes simplex	
	5-BDU	5-IDU	5-BDU	5-IDU
Thymidine, 200 μg/disc				
Discs 5 mm apart	0	0, 0	0	0
" 30 " "	17	9, 12	10	12
Deoxyuridine, 200 μg/disc; discs side by side	17	17	17	17
Control	15, 20, 15	15, 17	15, 17	15, 17

* Diameter measurements were not used since thymidine reversal was most effective on that portion of the plaque-free zone lying between the 2 test discs. The radii reported were measured from center of halide deoxyuridine discs to edge of plaque inhibition zone in the direction of the thymidine or deoxyuridine discs. Data for controls are for the shortest radii. Fig. 1 illustrates the necessity for this method of measurement.

The data presented here are not to be considered quantitative since variations in the culture vessels result in differences in size of plaque-free zones. Nevertheless, there was indication that BDU was somewhat more active than IDU, which would agree with the *in vitro* results of Prusoff(6) in studying inhibition of DNA biosynthesis in mouse tumor cells. For example, zones of plaque inhibition produced by IDU contained at times a number of residual plaques while zones produced by BDU seemed to be truely plaque-free. This difference was most noticeable with herpes simplex virus.

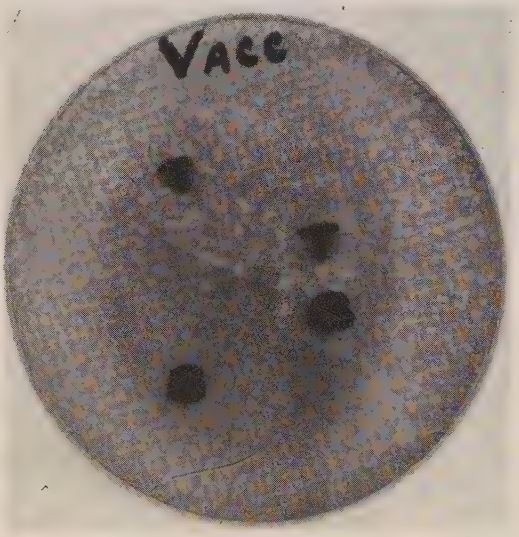

FIG. 1. Four day agar overlay culture containing plaques produced by vaccinia virus. B's cover discs with 20 μg of BDU, T's cover discs with 200 μg of thymidine. Note in the case of the pair of discs separated by 5 mm, to the right, that although plaques are well formed in the area of the discs some plaque suppression still occurs outside the area of thymidine diffusion (lower right of dish).

As might have been predicted from a previous report(1), when 200 μg of thymidine was placed in close proximity to 20 μg of IDU or BDU on a vaccinia or herpes simplex virus infected agar overlay culture, plaque inhibition was reversed (Table II, Fig. 1). When the distance between the thymidine disc and the halogen deoxyuridine discs was increased from 5 to 30 mm, plaque inhibition again was produced. There was some indication that even at 30 mm distance thymidine had some effect on IDU. This is an example of how media constituents could cause discrepancies in testing antiviral agents *in vitro*. If the media used here had contained significant levels of thymidine by some unusual circumstance, then plaque inhibition would not have been observed. It has been pointed out that FDU might be useful in determining nucleic acid content of various viruses(2). BDU and IDU, however, may be more reliable for this purpose.

Preliminary tests of IDU have been undertaken in vaccinia and herpes simplex infected eggs and animals, and there seemed to be some antiviral effect in eggs. The rapid breakdown of IDU in the animal body(6) could explain the apparent absence of antiviral activity in mouse tests. These *in vivo* studies will be reported later.

Summary. 5-Iodo- and 5-bromo-2′-deoxyuridine, when tested in the agar-diffusion plaque-inhibition test, were found to inhibit plaque formation of DNA containing vaccinia and herpes simplex viruses but not RNA containing West Nile and Newcastle disease viruses. A number of other compounds that might be candidates for the disruption of normal nucleic acid biosynthesis, including aminopterin and 5-fluoro-2′-deoxyuridine, also were tested and found inactive in this test system. Thymidine readily reversed plaque inhibition by both 5-iodo- and 5-bromo-2′-deoxyuridine.

1. Smith, J. D., Freeman, G., Vogt, M., Dulbecco, R., *Virology*, 1960, v12, 185.

2. Salzman, N. P., *ibid.*, 1960, v10, 150.

3. Tamm, I., *Clin. Pharmacol. and Therap.*, 1960, v1, 777.

4. Herrmann, E. C., Jr., Gabliks, J., Engle, C., Perlman, P. L., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 134.

5. Welch, A. D., Prusoff, W. H., *Cancer Chemotherapy Repts.* 1960, Issue 6, 29.

6. Prusoff, W. H., *Cancer Research,* 1960, v20, 92.

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Mammalian Degradation of (–)-Nicotine to 3-Pyridylacetic Acid and Other Compounds.* (26561)

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Data obtained from administration of randomly labelled nicotine-C^{14} in the rat(1) and the dog(2) have indicated that over 90% of the administered radioactivity is excreted in the urine as metabolites and as unchanged nicotine. Metabolic studies(3-5) in the dog with non-isotopic (–)-nicotine, and the metabolic intermediate (–)-cotinine have led to the isolation and characterization of metabolites which account for approximately 30% of the administered dose, *viz.*, (–)-cotinine, (–)-desmethylcotinine, hydroxycotinine, (+) - γ - (3 - pyridyl) - γ - methylaminobutyric acid, and γ-(3-pyridyl)-β-oxo-N-methylbutyramide. In addition, experiments in the rat(6) with nicotine-C^{14}-methyl have clearly shown a metabolism of the methyl group of nicotine to respiratory carbon dioxide and urea.

The foregoing, which suggest a complexity in the metabolism of nicotine far in excess of estimates by other authors(7), have led to further investigation of the metabolism of randomly labelled (–)-nicotine-C^{14}. It has now been demonstrated that a sequence of metabolic reactions leads from (–)-nicotine to 3-pyridylacetic acid.

Materials and methods. Two male mongrel dogs, 11.7 and 13.8 kg under pentobarbital anesthesia received randomly labelled (–)-nicotine-C^{14}‡, 4.25 mg/kg, total radioactivity 1.07 × 10^6 CPM intravenously over an 8-hour period. The urine was collected from the bladder by an indwelling catheter during infusion and a subsequent 19-hour period. Radioactive determinations were made on infinitely thick samples which were dried on talc. Urine samples were processed by modifications of previously described procedures(3-5) and descending paper chromatograms were prepared on Whatman No. 1 paper as previously described(3-5).

Studies on the metabolism of (–)-cotinine were conducted on an adult male mongrel dog. (–)-Cotinine was administered orally (100 mg/kg). The animal received commercial pellets and water *ad libitum*, and urine (preserved with sodium fluoride) was collected as run-off from the metabolism cage.

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‡ The authors are greatly indebted to Dr. L. J. Dewey and Dr. W. Stepka for this material which was extracted from *N. rustica* which was grown in an atmosphere of $C^{14}O_2$.

The daily urine samples were combined and frozen.

Results. The combined bladder urine from the 2 dogs which had received the radioactive nicotine contained 8.4×10^5 CPM, 79% of the administered radioactivity. The urine was then passed through a column of Dowex 50 (H^+) which served to retain basic material. The combined effluent and a water wash contained 3.7×10^4 CPM. The column was eluted with 4 M ammonia water to give a solution (fraction B) which contained 5.5×10^5 CPM, 65% of administered radioactivity. The column retained 3.03×10^5 CPM. This latter fraction was eluted with hydrochloric acid. Studies of the nature of the metabolites in this acidic eluate have been recorded elsewhere(8).

The ammoniacal solution (fraction B) was subjected to exhaustive extraction with chloroform to remove cotinine and other basic metabolites. The chloroform solution upon paper chromatography in the ammonia system showed Koenig positive radioactive zones corresponding to (–)-nicotine, (–)-cotinine, desmethylcotinine, hydroxycotinine, and some uncharacterized material. The aqueous solution, after extraction, showed by paper chromatography with both the formic acid and ammonia systems, radioactive and Koenig positive material corresponding by cochromatography to γ-(3-pyridyl)-γ-methylaminobutyric acid. A small amount of material corresponding in Rf value to cotinine, which could have arisen from spontaneous lactamization of the methylamino acid, was also disclosed. Other radioactive zones were not subjected to identification. One, however, corresponded in Rf value to authentic 3-pyridylacetic acid, authentic samples of which separated readily at Rf 0.12 from γ-(3-pyridyl)-γ-methylaminobutyric acid (Rf 0.15) in the ammonia system when concentrations of either of the 2 components were not excessive.

The residue obtained by evaporation of the foregoing chloroform solution was treated with water and the aqueous mixture was placed upon Dowex 1 (OH^-). The effluent contained basic components, cotinine, hydroxycotinine and desmethylcotinine. The column retained a variety of Koenig positive components, Rf 0.27, 0.33, 0.46, 0.60, 0.74 (ammonia system) and Rf 0.05, 0.21, 0.34, 0.46 (formic acid system). These substances were readily eluted from the column with dilute acetic acid. The zone at Rf 0.74 (ammonia system), Rf 0.34 (formic acid system) cochromatographed with authentic γ-(3-pyridyl)-β-oxo-N-methylbutyramide(4).

The combined urine (6-1) from the dog receiving 100 mg/kg of (–)-cotinine per day for 14 days was adjusted to pH 8-9 by addition of ammonia water and then continuously extracted with chloroform. The chloroform solution yielded by published procedures(3-5): (–)-desmethylcotinine (1.2 g, 5.3 molar % of the dose of (–)-cotinine), γ-(3-pyridyl)-β-oxo-N-methylbutyramide (1.06 g, 4.04%) and unchanged (–)-cotinine (2 g, 8.33%). Hydroxycotinine of the urine was converted by treatment with acetic anhydride-pyridine to the acetyl derivative(5) for isolation (4.64 molar %).

The extracted urine was then placed upon a column of Dowex 21K (OH^-) (4-1). The combined effluent and water wash contained Koenig positive material, disclosed with *p*-aminobenzoic acid and cyanogen bromide, which was not further investigated. The column was then eluted with 1 M acetic acid which served to remove Koenig positive components (Rf 0.02 and Rf 0.12, ammonia system; Rf 0.05 and Rf 0.41, formic acid system). Following treatment with decolorizing carbon, the eluate was evaporated to dryness. The residue was triturated with 25 ml of absolute ethanol and then filtered. After addition of acetone (25 ml), the solution was cooled and filtered. The residue from evaporation of the solvent was dissolved in dilute hydrochloric acid and at pH 2 placed upon a column of Dowex 50 (H^+) (1×10 cm). After a water wash, the column was eluted with 1 M ammonia water. The ammoniacal solution was placed on Dowex 21K (OH^-). After a water wash, the column was eluted with 25 ml of 1 M acetic acid. The residue from evaporation of the solvent was reprocessed on the 2 exchange resins as described above to give 300 mg of light-colored viscous oil, which gave Koenig positive zones at Rf 0.12 and 0.02 (ammonia system) and Rf 0.41 and 0.05 (formic acid system). The major zone (Rf 0.12 and Rf 0.41, respectively) corresponded in value to that of authentic 3-pyridylacetic acid, prepared according to Malon and Dean(9). The oil, upon treatment with picric acid in ethanol(4), yielded 250 mg of 3-carboxymethylpyridinium picrate, m.p. 167-168° after recrystallization from methanol. The analytical sample was dried at 60° and 1 mm over potassium hydroxide. Calculated for $C_{13}H_{10}N_4O_9$: C, 42.63; H, 2.75; N, 15.30. Found: C, 42.51; H, 2.93; N, 15.42.§ The melting point was not depressed upon admixture with authentic material(4), m.p. 167-168°.

The foregoing picric acid salt from the metabolic experiment was dissolved in water and placed on Dowex 50 (H^+). After removal of picric acid by a water wash, the column was eluted with 25 ml of 4 M ammonia water. The ammoniacal solution was placed on a column of Dowex 21K (OH^-). After a water wash, the column was eluted with 1 M acetic acid. The acidic solution yielded pure crystalline 3-pyridylacetic acid, m.p. 144-146°, upon evaporation of the solvent. For analysis, the compound was recrystallized from methanol and dried under diminished pressure, m.p. 144-146°. Calculated for $C_7H_7NO_2$: C, 61.32; H, 5.15; N, 10.22. Found: C, 61.45; H, 5.18; N, 10.24. The melting point was not depressed by admixture with an authentic sample, m.p. 144-146°.

Discussion. The pattern of excretion of radioactive metabolites of (–)-nicotine affords a confirmation and extension of earlier studies in which a high degree of urinary excretion of nicotine and metabolites has been noted. The screening procedure employed in the present study, which was based upon methods developed for isolation and identification of metabolites of nicotine (3-5) also reveals that the number of metabolites in urine far exceeds the original estimates(7) which pointed to 3 major and 4 minor metabolites. Moreover, the structural relationships of the metabolites isolated in the present and previous studies are in accord with a metabolic sequence which involves participation of pyridine compounds in excess of 7. In addition to these, the urine contains a variety of labelled compounds resulting from demethylation of (–)-cotinine and production of one-carbon metabolites as well as those which contain C^{14} as the result of loss of 2 of the labelled carbons in the pyrrolidine ring during degradation of (–)-nicotine to 3-pyridylacetic acid.

The isolation of 3-pyridylacetic acid, which has apparently never been previously recorded as a metabolic or natural product, serves to suggest a sequence: (–)-nicotine → (–)-cotinine → γ-(3-pyridyl)-β-oxo-N-methylbutyramide → γ-(3-pyridyl)-β-oxobutyrate + methylamine → 3-pyridylacetate + acetate. This interpretation is apparently consistent with the experiments of Werle and Meyer (10) in which nicotine was converted to methylamine in the presence of liver slices. Metabolic studies with γ-(3-pyridyl)-β-oxo-N-methylbutyramide and the corresponding acid are, however, required for substantiation or rejection of the hypothetical metabolic route.

Little is known of the metabolism of 3-pyridylacetic acid in the dog. The compound has been studied in the rat and the human (11). In both species following oral administration of 3-pyridylacetic acid a high degree of urinary excretion of the compound was reported. Ratti and De Fina reported(12) success in depressing serum cholesterol values in man by sustained oral administration of gram quantities of 3-pyridylacetic acid.

Summary. 1. The pattern of urinary excretion of C^{14} in the dog following intravenous administration of (–)-nicotine-C^{14} has been examined chromatographically by improved procedures. 2. The fractionated urine showed C^{14} activity corresponding chromatographically to the known metabolites: cotinine, γ-(3-pyridyl)-γ-methylaminobutyric acid, desmethylcotinine-hydroxycotinine, and γ-(3-pyridyl)-β-oxo-N-methylbutyramide. 3. Among many unidentified radioactive components, the urine contained material cor-

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§ Microanalyses by Spang Micronalytical Laboratory and by Weiler and Strauss.

responding chromatographically to 3-pyridylacetic acid. 4. Following oral administration of (−)-cotinine to the dog, 3-pyridylacetic acid was isolated from urine and characterized by analysis, melting point, mixed melting point and as its picric acid salt. 5. A precursor of 3-pyridylacetic acid is on formal grounds γ-(3-pyridyl)-β-oxobutyric acid, derivable from the known metabolite γ-(3-pyridyl)-β-oxo-N-methylbutyramide.

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1. Ganz, A., Kelsey, F. B., Geiling, E. M. K., *J. Pharmacol. Exp. Therap.*, 1951, v103, 209.

2. Bennett, D. R., Tedeschi, R. E., Larson, P. S., *Arch. Int. Pharmacodyn.*, 1954, v98, 221.

3. McKennis, H., Jr., Turnbull, L. B., Bowman, E. R., *J. Am. Chem. Soc.*, 1958, v80, 6597.

4. McKennis, H., Jr., Bowman, E. R., Turnbull, L. B., *ibid.*, 1960, v82, 3974.

5. McKennis, H., Jr., Turnbull, L. B., Bowman, E. R., Wada, E., *ibid.*, 1959, v81, 3951.

6. McKennis, H., Jr., Wada, E., Bowman, E. R., Turnbull, L. B., in press.

7. Owen, F. B., Jr., Larson, P. S., *Arch. Int. Pharmacodyn.*, 1958, v115, 402.

8. Turnbull, L. B., Bowman, E. R., McKennis, H., Jr., *Fed. Proc.*, 1960, v19, 268.

9. Malon, R. L., Dean, P., *J. Am. Chem. Soc.*, 1947, v69, 1797.

10. Werle, E., Meyer, D., *Biochem. Z.*, 1950, v321, 221.

11. Ginoulhiac, E., Tenconi, L. T., *Min. Med.*, 1959, v5, 1166.

12. Ratti, G., De Fina, E., *Lancet*, 1959, v2, 917.

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Immunoassay of Insulin Content of Crystalline Glucagon Preparations.

(26562)

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Purification and crystallization of glucagon (1) have resulted in a preparation of exceedingly high purity. However, since no specific procedure for inactivation of insulin is included in the purification procedure, a slight insulin contamination is virtually unavoidable. It has therefore been suggested(2) that results obtained in biological experiments with glucagon should be interpreted with caution particularly when large doses of glucagon are used. McNaught(3), by measuring glucose uptake and net gas output by slices of lactating rat mammary gland estimated the insulin content of a glucagon preparation to be about 1.0%. Randle(4) determined glucose uptake by rat diaphragm and found the insulin content of an amorphous glucagon preparation to be 1.2%. One crystalline preparation contained less insulin but another (Lot #258-234 B-54-2) produced an even greater increase in glucose uptake than did the amorphous glucagon at the same concentration. J. R-Candela and R. R-Candela (5) reported that glucagon reduced the effect of insulin on glucose uptake and Snedecor *et al.*(6) found a similar inhibition of insulin-induced glycogen deposition. In contrast, Narahara and Williams(7) reported that glucagon potentiates the action of small doses of insulin on diaphragm. Recently, Lee and associates(8) estimated the insulin content of several crystalline glucagon preparations to range from 0.007% to 0.08% by measurement of $C^{14}O_2$ production from glucose-1-C^{14} in the rat epididymal fat pad preparation.

In view of the uncertain effects of glucagon on the response of tissues to insulin and the marked differences in estimated insulin content of crystalline glucagon preparations reported by various investigators an immunologic assay for insulin in 2 glucagon preparations (lots #258-234 B-54-2 and lot #258-234 B-167-1)* was undertaken, the results of which are recorded herein.

Methods. The immunoassay employed here depends on the ability of insulin in unknown solutions to inhibit, competitively, the

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* We are indebted to Dr. O. K. Behrens, Lilly Research Laboratories, for supplies of crystalline glucagon.

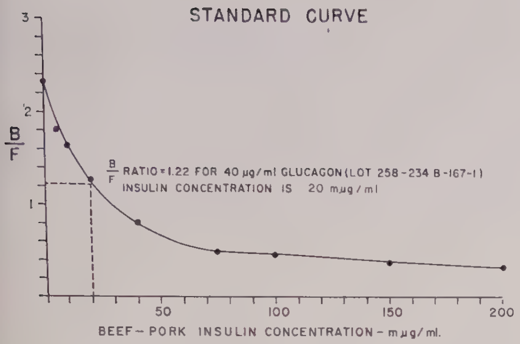

FIG. 1. Ratio of antibody-bound beef-insulin-I^{131} to free insulin-I^{131} (B/F) as a function of concentration of added beef-pork insulin. Ratios were obtained from the complete series of chromatoelectrophoretograms, a few of which are shown on the left side of Fig. 2. The method for determination of insulin content of an unknown sample yielding a B/F ratio of 1:22 is indicated.

combination of I^{131}-labeled insulin with insulin-binding antibodies(9). Degree of reduction in the ratio of antibody-bound insulin-I^{131} (B) to unbound ('free') insulin-I^{181} (F) is a function of the insulin concentration. Separation of bound and free insulin is effected by paper chromatoelectrophoresis wherein the free insulin is adsorbed at site of application(10,11) and antibody-bound insulin migrates with the serum proteins(11).

For preparation of a standard curve, trace amounts of crystalline beef insulin-I^{131} are incubated with known amounts of unlabeled crystalline insulin at various concentrations and with antiserum in fixed concentration. For determination of the insulin content of an unknown solution, an aliquot of the latter is substituted for the insulin employed in the standard solutions. Human serum albumin (2.5 mg/ml) is added to all solutions to prevent losses of insulin by adsorption to glassware. After 4 days at 4°C the mixtures are applied to Whatman 3 MM paper strips for chromatoelectrophoresis(11). The strips are scanned for radioactivity with an automatic recorder and the areas under the bound and free peaks determined with a planimeter. The B/F ratios in standard solutions are plotted against the known insulin concentrations (Fig. 1). The insulin content of an unknown sample is obtained by reference to the standard curve for the concentration corresponding to the observed B/F ratio.

Preparation of insulin-I^{131} and other technical aspects have been described(11,12).

Most antiserums show quantitative differences in reaction with insulins from different animal species(13). Therefore the present study employed a human antiserum (L.J.) to beef-pork insulin which had previously (13) been shown to react very similarly with beef and pork insulins. Since the glucagon preparations tested are obtained from mixtures of beef and pork pancreatic extracts, the precise contribution of each being unknown, standard curves were prepared with 2 sets of insulin solutions, one containing equal parts crystalline beef insulin and crystalline pork insulin, the other containing pure beef insulin. Since beef insulin reacts slightly more strongly than pork insulin with antiserum L.J., the insulin content of glucagon estimated from the standard beef insulin curve represents a minimum estimate whereas that estimated from the beef-pork insulin standards is probably more realistic.

Both glucagon preparations were analyzed for insulin at concentrations of 20, 30, 40, 50 and 75 μg/ml. In addition, Lot #258-234 B-167-1 was treated with 0.02 M cysteine at pH 8.0 for 12 hours at 37°C to inactivate insulin (14) and was then dialyzed against distilled water for 24 hours to remove the cysteine.

Results. Radioactive scans of the paper strips are shown in Fig. 2 for several of the beef-pork insulin standards and for the glucagon series of Lot #258-234 B-167-1 with and without cysteine treatment. Insulin concentrations determined by reference to the beef-pork insulin and to the beef-insulin standard curves for both glucagon preparations are given in Table I. None of the cysteine-treated mixtures contained any immunologically active insulin. Glucagon Lot #258-234 B-54-2 contained 0.041 ± 0.003% insulin and glucagon Lot #258-234 B-167-1 contained 0.048 ± 0.006% insulin if nearly equal amounts of beef-pork insulin were present. If it is assumed that the preparations contained beef insulin exclusively the results would indicate insulin contents 12% and 10% lower respectively. However, the data themselves provide a better fit to a beef-pork insulin than to a beef insulin standard curve.

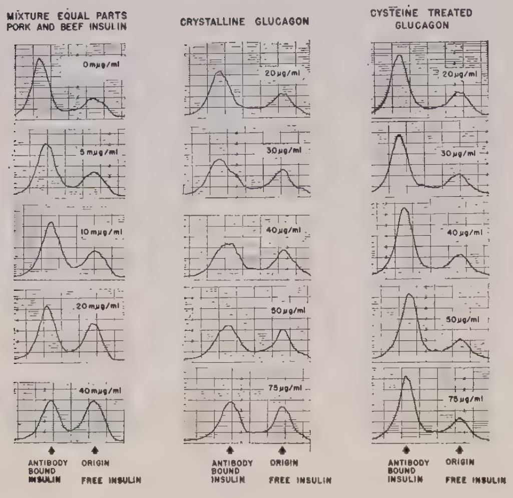

FIG. 2. Radiochromatoelectrophoretograms of mixtures containing trace concentrations of beef insulin-I^{131} and standard solutions of crystalline beef-pork insulin (left), crystalline glucagon (middle) and cysteine treated glucagon (right).

Discussion. Because of possible modifying effects of glucagon on response to insulin in tissues, measurement of the minute insulin contaminant of glucagon by bioassay remains open to some question. Since cysteine has no effect on glucagon itself(8), the absence of any effect of cysteine-treated glucagon preparations on the B/F ratios for insulin-I^{131} provides evidence that glucagon neither augments nor inhibits the reaction of insulin with insulin-binding antibodies; nor would such interference be anticipated in view of the known specificity of antigen-antibody reactions and the demonstration that plasma proteins do not affect the immunoassay of insulin(12).

Although our results are lower than some of those reported previously with tissue assays, the results of Lee *et al.*(8) are lower still, being 0.013% (confidence limits 0.0058%-0.030%) for Lot #258-234 B-54-2. Although these authors could not entirely exclude an effect of glucagon in their system, the cysteine-treated insulin still exhibiting a slight amount of insulin-like activity, the discrepancy in results between the fat pad assay and the immunologic assay cannot be resolved on this basis alone since correction for insulin-like activity of glucagon would have reduced still further the insulin content estimated by the fat pad assay. However, since, in addition, the slope of concentration $C^{14}O_2$ response curve differed for insulin and glucagon preparations, it is possible that glucagon alters the fat pad response to insulin. In any event it appears that the insulin content of the glucagon preparations examined here is significantly less than 0.1%.

Summary and conclusions. 1. The insulin content of each of 2 crystalline glucagon preparations was found to be less than 0.05% by immunochemical assay. 2. Immunologically reactive insulin was not detected in a cysteine treated glucagon preparation.

TABLE I.

		Insulin concentration			
		From beef insulin standard curve		From beef-pork insulin standard curve	
Lot	Glucagon conc., μg/ml	mμg/ml	%	mμg/ml	%
258-234 B-54-2	20	5.5	.028	8.4	.042
	30	8.0	.027	12.0	.040
	40	14.0	.037	17.5	.044
	50	22.0	.044	22.0	.044
	75	32.0	.043	28.0	.037
			.036 ± .006*		.041 ± .003
258-234 B-167-1	20	8.0	.040	11.3	.056
	30	9.3	.031	12.8	.043
	40	19.8	.047	21.5	.054
	50	26.0	.052	25.0	.050
	75	35.0	.047	30.0	.040
			.043 ± .007		.048 ± .006
258-234 B-167-1	cysteine treated—no detectable insulin				

* Mean ± S.D.

1. Staub, A., Sinn, L., Behrens, O. K., *J. Biol. Chem.*, 1955, v214, 619.
2. Berthet, J., *Am. J. Med.*, 1959, v26, 703.
3. Randle, P. J., *J. Endocrinol.*, 1958, v17, 396.
4. McNaught, M. L., *ibid.*, 1958, v17, XVI.
5. R-Candela, J. L., R-Candela, R., *Ciba Foundation Colloquia Endocrinol.*, 1956, v9, 194.
6. Snedecor, J. G., DeMeio, R. H., Pincus, I. J., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 396.
7. Narahara, H. J., Williams, R. H., *J. Biol. Chem.*, 1958, v233, 1034.
8. Lee, H. M., Ellis, R. M., Bromer, W. W., PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 4.
9. Berson, S. A., Yalow, R. S., *Advances in Biol. & Med. Physics,* Acad. Press, 1958, v6, 349.
10. Kallee, E., *Z. f. Naturforsch.*, 1952, v7b, 661.
11. Berson, S. A., Yalow, R. S., Bauman, A., Rothschild, M. A., Newerly K., *J. Clin. Invest.*, 1956, v35, 170.
12. Yalow, R. S., Berson, S. A., *ibid.*, 1960, v39, 1157.
13. Berson, S. A., Yalow, R. S., *ibid.*, 1959, v38, 2017.
14. DuVigneaud, V., Fitch, A., Pekarek, E., Lockwood, W. W., *J. Biol. Chem.*, 1931, v94, 233.

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Metabolic Adaptations in Higher Animals VI. Liver Arginase Activity During Adaptation to High Protein Diet.* (26563)

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Changes in liver arginase activity that represent adaptive responses to dietary changes have been reported from several laboratories. Liver arginase activity per unit of tissue decreases when rats are fed low-protein or protein-free diets(1-4) and increases when they are fed a high-protein diet(4) or are starved (5).

Lightbody and Kleinman(6) first observed that liver arginase activity was responsive to protein intake. They reported that activity per unit of dry liver or per unit of body weight increased with each increase in the dietary level of protein in rats fed on diets containing 6, 25, 60 and 75% protein. Mandelstam and Yudkin(7) also showed that liver arginase activity, per unit of liver or per unit of body weight, in rats was proportional to the amount of protein in their diet and that the response to protein was linear for rats fed on diets containing 20, 40 and 60% of protein. However, Maramatsu and Ashida(8) reported that, although this was true of rats fed on diets containing more than 20% of protein, it did not hold for those fed on diets containing less than 20%.

Little has been reported on the relationship between total arginase activity and amount of urea excreted. A high positive correlation between liver arginase activity and urea excretion was found in an experiment on rats fed for 2 weeks on diets containing 0, 5, 10, 15, 25, 40, and 60% of casein(8), but the results of Rosenthal and Vars(5) who used protein-depleted rats subjected to 2 and 4 days of starvation do not reveal such a correlation. There is also a lack of information about the time-course of the change in arginase activity in response to a change in diet. Mandelstam and Yudkin(7) measured activity at weekly intervals and observed that the response to a change in dietary level of protein reached a maximum within 2 to 3 weeks.

Recently Freedland and Harper(9) reported that a substantial response in liver glucose-6-phosphatase activity occurred within 24 hours after fructose or protein was substituted for "dextrin" in the diet of rats. Therefore, the adaptation of liver arginase during the first few days after feeding rats a high-protein diet has been investigated and the

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relationship between total arginase activity and amount of urea in the urine during the adaptation period has been determined. The latter was of particular interest because the food intake of rats falls for one or 2 days when the protein content of their diet is greatly increased. The effect of a high protein intake on activity of kidney arginase was also measured.

Experimental procedure. Animals and diets. Male Holtzman rats weighing from 60 to 65 g were used in all experiments. The 3 different diets used throughout this study were as follows: (1) a control diet containing 64.6% of dextrin (moist cornstarch heated in an autoclave at 115° for 2 hours, then dried and ground) and 25% of casein; (2) a diet containing 44.35% of dextrin and 45% of casein; (3) a diet containing 19.1% of dextrin and 70% of casein. All diets contained 4.5% of corn oil, 5% of mineral mixture, 0.15% of choline chloride, and 0.5% of fat-soluble vitamin mixture in corn oil(10). A mixture of water-soluble vitamins in sucrose(10) was added as follows: to the 25% casein diet, 0.25%; to the 45% casein diet, 0.5%; to the 70% casein diet, 0.75%.

Rats used to study the change in arginase activity with time were fed *ad libitum*. The food intake of those used to study the relationship between arginase activity and urea excretion was restricted and the groups were pair-fed. Each rat was placed in an individual metabolism cage and urine was collected in dilute sulphuric acid for 2 days following the first, 5th, and 9th days after the casein content of the diet had been increased from 25% to 70%. The funnels were rinsed with distilled water at the end of each period and the urine was stored at 4°C.

When the effect of changing the casein content of the diet from 70% to 25% was studied, the animals were fed the 70% casein diet for 8 days, then were divided into 2 groups; the first group was continued on the 70% casein diet and served as a control, the second was offered the 25% casein diet.

All rats were fed the control diet for 4 days from the time they were received until they were offered the experimental diets, to compensate for any lack of uniformity in previous dietary history. Rats were weighed, either daily, or every other day, depending upon type of experiment.

Arginase activity. Animals were killed at intervals after being given the experimental diets as indicated in the results. The livers, and in some cases the kidneys, were removed, weighed, and placed in ice. A portion of the chilled liver or kidney was homogenized in 9 volumes of water and filtered through cheesecloth. The liver homogenate was diluted 100-fold with water and the kidney homogenate 10-fold. One portion of the diluted homogenate was used for the arginase assay, and another for determination of protein. Arginase activity was measured by the method of Greenberg(11) without activation, because, in another series of experiments, arginase of homogenates from livers of rats fed the control or the high protein diet was found to be activated in proportion to the initial activity. For the enzyme determination, 0.5 ml of 5% arginine solution, pH 9.5, was added to 0.5 ml of the 0.1% homogenate of liver or to 1 ml of the 1% homogenate of kidney, both of which had been incubated at 25°C for 3 minutes. After incubation at the same temperature for 5 minutes, the reaction was stopped by addition of 1 ml of glacial acetic acid. Then the activity was estimated by measuring the amount of urea produced(12). A portion of the diluted homogenate was dissolved in 1 N sodium hydroxide and protein content was estimated by the method of Lowry *et al.*(13).

The amount of urea in urine was determined by the method used for the estimation of arginase activity(12).

Results. Arginase activity is expressed as micromoles of urea formed in one minute at 25°C and pH 9.5 per mg of liver protein nitrogen (specific activity) or per g of body weight.

Change in liver arginase activity after feeding high-protein diets. Changes in liver arginase activity 1, 2, 4, 7, and 10 days after increasing the casein content of the diet are shown in Fig. 1 and 2. Liver arginase activity of rats increased within one day when the casein content of the diet was increased from 25% to 45 or 70% whether the results were expressed as activity per mg of liver protein

TABLE I. Effect of Increasing Protein Content of Diet on Food Consumption.

Diet	Food consumption, g/rat						
	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
25% casein (control)	10.0 ± .4* (10)†	12.6 ± .4 (8)	13.5 ± .2 (6)	14.0 ± .6 (6)	13.8 ± 1.8 (4)	15.5 ± .4 (4)	15.2 ± .4 (4)
45% casein	7.1 ± .2 (20)	9.7 ± .2 (16)	11.3 ± .4 (12)	12.3 ± .5 (12)	11.5 ± .5 (7)	13.8 ± .4 (7)	13.2 ± .5 (7)
70% casein	5.6 ± .3 (10)	7.2 ± .3 (8)	8.1 ± .2 (6)	9.4 ± 1.2 (6)	9.7 ± .2 (4)	11.1 ± .4 (4)	10.2 ± .3 (4)

* Stand. error of mean. † Numbers in parentheses show No. of animals used.

nitrogen (specific activity) (Fig. 1) or per g of body weight (Fig. 2). The activity per g of body weight of the group receiving 45% of casein continued to increase for 4 days and that of the group receiving 70% of casein continued to increase for 7 days. For each group and at each time interval the increase in arginase activity per unit of body weight was much greater than the increase per unit of liver nitrogen. This occurred because the livers of rats fed the high protein diets were larger than those of rats fed the control diet. Similar effects of a high protein intake on glucose-6-phosphatase activity were observed by Freedland and Harper(14).

Effect of high protein diet on body weight gain and food consumption. Growth curves for rats receiving the high protein diets are shown in Fig. 3. Growth was retarded for one day when the casein content of the diet was increased to 45%, and for 2 or 3 days when it was increased to 70%, thereafter growth rates of the 3 groups were similar. The slightly lower gain of the control group for one day may have been due to the rats becoming disturbed when they were redistributed in order to equalize the average body weights of the groups.

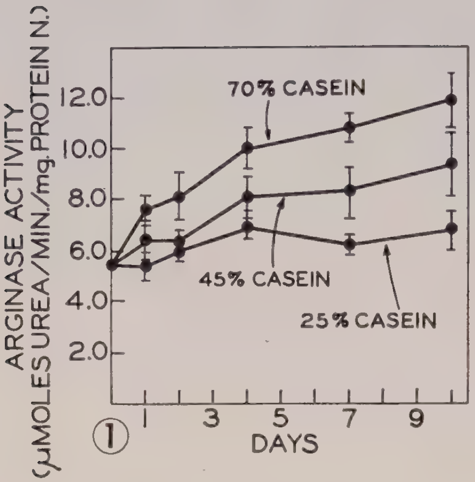
The food consumption figures in Table I show that there was an inverse relationship between protein content of the diet and food intake. Thus caloric intake was depressed for a few days after the protein content of the diet was raised.

Loss of arginase activity after changing from a high protein diet to a low protein diet. The results presented in Fig. 4 represent the change in liver arginase activity per unit of body weight when the diet was changed from 70% casein to 25% casein. Activity of liver arginase decreased, rate of loss being almost the inverse of rate of increase observed when the diet was changed from 25% casein to 70% casein.

Effect of changing from a high protein diet to a low protein diet on body weight gain and food consumption. The body weight gain for one day when the diet was changed from 70% casein to 25% casein was well above that of rats fed 70% casein diet, and thereafter growth rates in both groups were almost the same (Fig. 5). Food consumption of the former on the day when the diet was changed was also above that of the latter, 13.3 ± 1.0 g. compared to 11.3 ± 0.2 g. The slight growth retardation observed in the 70% casein group for one day may have been due to the animals being disturbed when the groups were redistributed as mentioned previously (Fig. 3).

Relationship between liver arginase activity and urinary urea production. Rats that were fed the control diet (25% casein) for 4 days were divided into 2 groups; one was fed the 70% casein diet and the other was continued on the control diet. The amount of each diet fed was restricted to approximately the amount consumed by the 70% casein group under *ad libitum* feeding conditions (Table I) so that each group would have the same caloric intake. The quantities fed daily from the first to the tenth days were as follows: 5, 7, 8, 9, 9.5, 10, 11, 12, 13 and 13 g. Rats were killed 2, 6, and 10 days after changing diet and urine was collected for 2 days before killing rats. Weight gains of the two groups were almost the same (Table II) but average liver weight of the group fed the 70% casein diet was significantly greater than that of the group fed the 25% casein diet.

The relationship between total liver argi-



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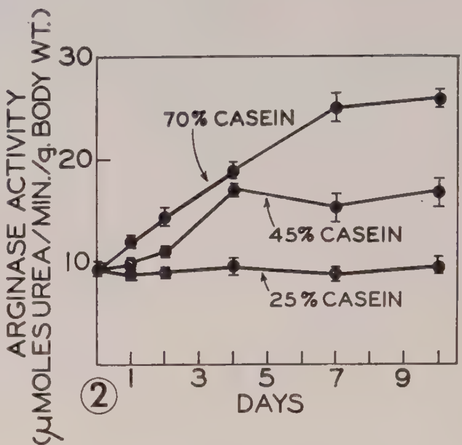

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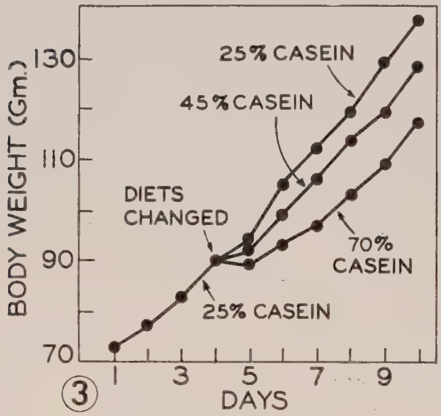

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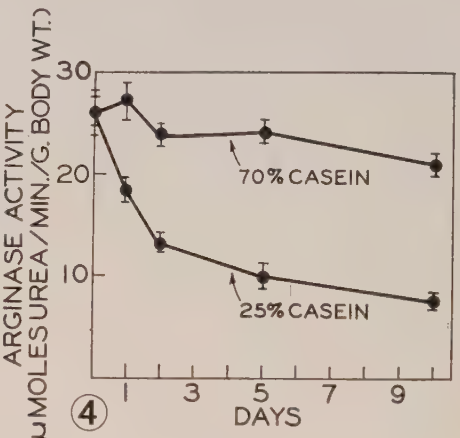

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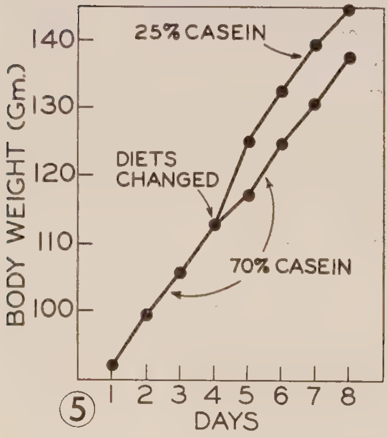

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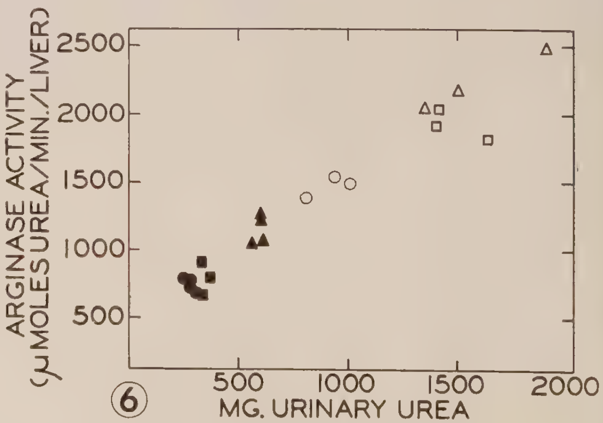

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FIG. 1. Change in liver arginase activity after feeding high protein diets. Values expressed as activity per mg of liver protein nitrogen (specific activity). Each value is the average for 4 to 7 animals. Vertical lines represent standard errors of means.

FIG. 2. Change in liver arginase activity after feeding high protein diets. Values expressed as activity per g of body weight. Each value is the average for 4 to 7 animals. Vertical lines represent standard errors of means.

TABLE II. Comparison of Liver Weights of Rats Fed Equal Quantities of Medium or High Protein Diets.

Days after changing diet	Body wt, g		Liver wt, g	
	25% casein	70% casein	25% casein	70% casein
2	84 ± 2*†	83 ± 2	3.18 ± .22	4.89 ± .32
6	105 ± 5	102 ± 4	4.08 ± .17	5.93 ± .07
10	132 ± 1	131 ± 3	5.05 ± .10	6.57 ± .20

* Stand. error of mean. † Each value represents avg for 4 animals.

TABLE III. Effect of High Protein Diet on Kidney Arginase Activity and Kidney Weight of Rats Having Equal Food Intakes.*

			Kidney arginase activity			
Days after changing diet	Kidney wt, g		Urea produced per mg of kidney nitrogen, μmol		Urea produced per mg of body wt, μmol	
	25% casein	70% casein	25% casein	70% casein	25% casein	70% casein
2	.86 ±.03†	1.09 ±.03	.50 ±.06	.49 ±.02	.13 ±.01	.17 ±.01
6	1.08 ±.03	1.22 ±.03	.42 ±.04	.62 ±.04	.11 ±.01	.21 ±.01
10	1.21 ±.04	1.60 ±.06	.41 ±.02	.69 ±.09	.09 ±.01	.22 ±.01

* Each value represents avg for 4 animals. † Stand. error of mean.

nase activity and amount of urea excreted in the urine is shown in Fig. 6. Throughout the experimental period total liver arginase activity increased linearly with increasing urinary urea excretion.

Change in kidney arginase activity after feeding high protein diet. The activity of kidney arginase was also measured in rats used in the experiment on the relationship between activity of liver arginase and amount of urea in urine. The results are shown in Table III. The activity of kidney arginase per mg of kidney protein and also per g of body weight increased between the second and the sixth day in rats fed the 70% casein diet. The kidneys of rats fed the 70% casein diet increased in size in about the same proportion as their livers.

Discussion. The time-course of the adaptive response of liver arginase in rats fed a high protein diet has been followed and a relationship between response of the enzyme and response of the whole animal to a high protein intake has been demonstrated.

Within one day after the protein content of the diet was raised from 25% to 45 or 70% food intake of rats fed the high protein diets was depressed and their growth was retarded. Within 24 hours liver arginase activity per unit of liver protein increased. Since a reduction in caloric intake results in an increase in liver arginase activity(15) this rise may have been due in part to the lowered food intake. However, the activity of the enzyme continued to increase for between 7 and 10 days and during this time liver size also increased, an effect not seen in rats suffering caloric restriction, so that by the end of one week arginase activity per unit of body weight had increased 2.5-fold. Thus the adaptation involved both increased liver size and increased enzyme activity. As a metabolic adaptation should result in a change in the activity of a system on a body weight basis, the adaptation to the

FIG. 3. Effect of high protein diet on body weight gain. Each value up to the fourth day is average for 24 animals; each thereafter is average for 8 animals.

FIG. 4. Loss of arginase activity after changing from high protein to low protein diet expressed per g of body weight. Each value is average for 4 to 6 animals. Vertical lines represent standard errors of means.

FIG. 5. Effect of changing from high protein to low protein diet on body weight gain.

FIG. 6. Relationship between total liver arginase activity and urinary urea production. ●, 25% casein group. ○, 70% casein group 2 days after changing diet; ■, 25% casein group, □, 70% casein group 6 days after changing diet; ▲, 25% casein group, △, 70% casein group 10 days after changing diet.

45% casein diet would appear to be complete within 4 days and that to the 70% casein diet within 7 days (Fig. 2).

By the second day, food intake and rate of growth of rats fed the high protein diets increased. Between the 7th and 10th days after the protein content of the diet had been raised, the rats receiving the high protein diets gained weight nearly as rapidly as the control rats receiving the 25% casein diet. Of particular interest in relation to this response is the observation that, for rats fed either 25% or 70% of casein and having the same caloric intake, the relationship between liver arginase activity and urinary urea excretion was nearly linear throughout the entire adaptation period.

Although arginase would not appear to be the most limiting of the urea cycle enzymes when their activities are measured *in vitro* (16), these observations indicate that activity of liver arginase is proportional to extent of protein catabolism in the body over the range of dietary protein levels studied. A further point indicating that the high enzyme activity represents an adaptive response of the animal to a high rate of protein catabolism is that within one day after the protein content of the diet was lowered liver arginase activity fell sharply. Although the evidence is indirect, these observations suggest that food intake is depressed when protein content of the diet is raised because the animal is unable to metabolize an excess of protein until it undergoes various adaptations, one of which is a substantial increase in arginase activity.

Summary. 1. Liver arginase activity of rats increased within one day when the casein content of their diet was increased from 25% to 45% or 70% whether the results were expressed as activity per liver or per g of liver protein nitrogen. Arginase activity of the group receiving 45% casein continued to increase for 4 days and that of the group receiving 70% casein continued to increase for 7 days. Average liver weight of the high protein groups also increased significantly above that of the 25% casein group. 2. Growth rate and food consumption were depressed for one day when the casein content of the diet was increased to 45%, and for 2 or 3 days when it was increased to 70%; thereafter, growth rates of the 3 groups differed only slightly. 3. When the casein content of the diet was dropped from 70% to 25% and rate of loss of arginase activity was plotted graphically the curve obtained was almost the reverse of that obtained when the casein content of the diet was raised from 25% to 70%. 4. A correlation between total activity of liver arginase and amount of urea in urine was observed throughout 10 days after changing the casein content of the diet from 25% to 70%. 5. Kidney arginase activity and kidney weight also increased when casein content of the diet was raised from 25% to 70%.

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1. Ashworth, U. S., PROC. SOC. EXP. BIOL. AND MED., 1939, v42, 145.

2. Seifter, S., Harkness, D. M., Rubin, L., Muntwyler, E. J., *J. Biol. Chem.,* 1948, v176, 1371.

3. Rosenthal, O., Rogers, C. S., Vars, H. M., Ferguson, C. C., *ibid.,* 1950, v185, 669.

4. Miller, L. L., *ibid.,* 1950, v186, 253.

5. Rosenthal, O., Vars, H. M., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 555.

6. Lightbody, H. D., Kleinman, A., *J. Biol. Chem.,* 1939, v129, 71.

7. Mandelstam, J., Yudkin, J., *Biochem. J.,* 1952, v51, 681.

8. Muramatsu, K., Ashida, K., *J. Agr. Chem. Soc. Japan,* 1957, v31, 607.

9. Freedland, R. A., Harper, A. E., *J. Biol. Chem.,* 1958, v233, 1.

10. Harper, A. E., *J. Nutrition,* 1959, v68, 405.

11. Greenberg, D. M., in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology,* vII, Academic Press, Inc., New York, 1955, p. 368.

12. Engel, M. G., Engel, F. L., *J. Biol. Chem.,* 1947, v167, 535.

13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, B. J., *ibid.,* 1951, v193, 265.

14. Freedland, R. A., Harper, A. E., *ibid.,* 1957, v228, 743.

15. Muramatsu, K., Taga, Y., Ashida, K., *J. Agr. Chem. Soc. Japan,* 1957, v31, 603.

16. Dixon, M., Webb, E. C., *Enzymes,* Academic Press, Inc., New York, 1958, p642.

Received March 20, 1961. P.S.E.B.M., 1961, v107.

Inhibitory Actions of β-Diethylaminoethyl Diphenyl-n-Propyl Acetate (SKF 525-A) on Muscle Contraction. (26564)

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It has been known for several years that β-diethylaminoethyl diphenyl-n-propyl acetate (SKF 525-A) has an inhibitory action on the microsomal enzymes concerned with detoxification of foreign compounds. SKF 525-A has been shown to prolong the duration of action of a number of drugs which are detoxified by various diverse pathways; for example, by deamination, by N-demethylation, by side chain oxidation, by hydroxylation. These effects were reviewed by Brodie(1). SKF 525-A also inhibits some non-microsomal reactions including the formation of glucuronides and de-esterification of procaine. It has been shown to inhibit the soluble enzyme nitro-reductase(1).

The literature has been solely concerned with observations on the effects of SKF 525-A on enzyme systems; no work has been reported on the effects of SKF 525-A acting directly on skeletal and smooth muscle. The experiments to be described here are concerned with the effects of SKF 525-A on the isolated frog rectus abdominis muscle, the isolated rabbit ileum, and the frog gastrocnemius muscle.

Methods. (a) The rectus abdominis muscle of the frog (*Rana pipiens*) was suspended in an isolated organ bath containing frog Ringer's solution consisting of 111 mM NaCl, 1.9 mM KCl, 1.1 mM $CaCl_2$, 2.4 mM $NaHCO_3$.

(b) Segments of washed rabbit ileum were suspended in oxygenated Tyrode's solution containing 137 mM NaCl, 2.7 mM KCl, 0.90 mM $CaCl_2$, 1.06 mM $MgCl_2$, 11.9 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 5.5 mM dextrose. The solution was kept at 37°C, and contractions of the muscle were recorded on a kymograph drum using an ink recorder.

(c) The femoral artery of a pithed frog was cannulated just below the bifurcation of the aorta with a polyethylene cannula. The sciatic nerve was exposed and shielded electrodes were attached to it. The nerve was stimulated with square wave impulses of 1 millisecond duration at a frequency of 2 per second. Silver wire electrodes were embedded at each end of the gastrocnemius muscle, and the muscle was stimulated with square wave impulses of 20 milliseconds duration at a frequency of 2 per second. The threshold voltage which caused muscle contraction was measured for both nervous and muscle stimulation. Drugs were introduced via the polyethylene cannula.

(d) The effect of SKF 525-A on cholinesterase activity was measured as follows: 5 ml of human blood plasma were diluted with 5 ml sodium barbital solution (0.04 M) adjusted to pH 9.0. Ten ml of a 1% acetylcholine iodide solution were added and the volume made up to 50 ml with distilled water or with the solution under test. Changes in pH were measured at 1 minute intervals using a glass electrode.

Results. (a) *Frog rectus abdominis muscle.* SKF 525-A in a concentration of 500 μg/ml caused a slow steady contraction of the muscle which took 5 minutes to reach its peak. After this treatment the muscle was completely unresponsive to acetylcholine (ACh), and ACh response could not be restored even after repeated washings with fresh Ringer's solution (Fig. 1). Doses of 10 μg ACh/ml were given and the height of contraction observed in the presence of different concentrations of SKF 525-A. 0.1 μg/ml had no effect whereas 1.0 μg caused a 30% inhibition, and 100 μg caused complete inhibition of ACh-induced contractions.

The inhibition of the ACh effect by SKF 525-A was compared with that produced by d-tubocurarine. When SKF 525-A in a concentration of 10 μg/ml was added at the height of an ACh-induced contraction, no

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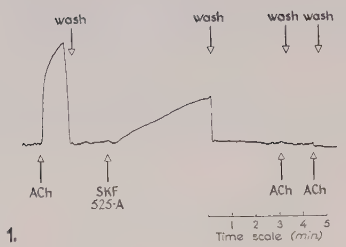

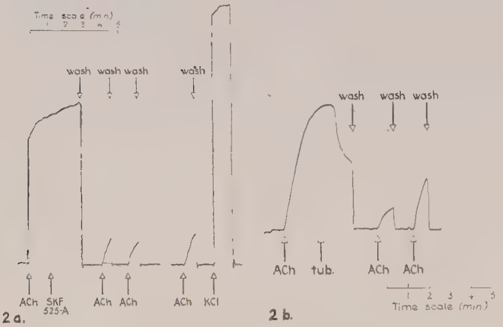

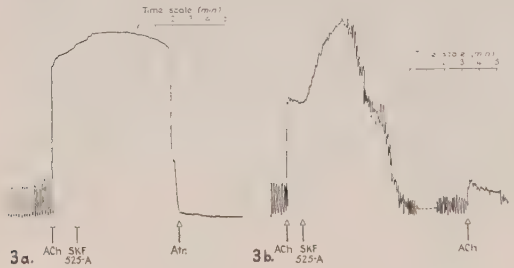

FIG. 1. Isotonic contractions of isolated frog rectus abdominis muscle in presence of ACh (10 μg/ml) and of SKF 525-A (500 μg/ml).

FIG. 2a. Isotonic contractions of isolated frog rectus abdominis muscle in presence of ACh (10 μg/ml), SKF 525-A (10 μg/ml), and potassium chloride (2%). 2b. Same after ACh (10 μg/ml) and d-tubocurarine (10 μg/ml).

FIG. 3a. Contractions of isolated rabbit ileum in presence of ACh (0.3 μg/ml), SKF 525-A (0.15 μg/ml), and atropine sulfate (0.15 μg/ml). 3b. Same after ACh (0.3 μg/ml) and SKF 525-A (1.5 μg/ml).

immediate relaxation occurred as was found with the same dose of d-tubocurarine (Fig. 2a, b). It was also observed that a muscle previously treated with SKF 525-A (10 μg/ml) was still refractory to ACh after being suspended in an eserine solution (5 μg/ml) for 20 minutes, whereas a muscle previously treated with d-tubocurarine and then suspended in the eserine solution responded to ACh after 20 minutes. After complete inhibition with SKF 525-A the muscle still contracted maximally in presence of a 2% KCl solution (Fig. 2a).

Procaine, a compound with a structure similar to SKF 525-A, was tested on the muscle preparation and found to have no effect on its response to ACh. The effect of atropine sulphate was studied. A concentration of 13 μg/ml produced rhythmic contractions of the muscle with increasing tension. After rinsing with fresh Ringer's solution the muscle was insensitive to ACh (10 μg/ml). In low doses atropine sulphate neither caused rhythmic contractions nor inhibited the ACh response.

(b) *Rabbit ileum.* 0.15 μg/ml SKF 525-A caused initially a slight potentiation of a contraction induced by ACh. On the other hand atropine in a similar concentration caused relaxation of the ileum and also stopped the peristaltic waves. A larger dose of SKF 525-A (1.5 μg/ml) caused a marked potentiation of the ACh contraction followed by relaxation. The muscle though still exhibiting spontaneous movement was now almost insensitive to ACh (Fig. 3a, b).

(c) *Frog gastrocnemius muscle.* When SKF 525-A (100 μg/ml) was injected in 0.2 ml doses into the femoral artery, the threshold to sciatic nervous stimulation rose. However, further experiments indicated that the effect was seen not only when the nerve was stimulated but also when the muscle was stimulated directly. Subsequently it was found that if the response to nervous stimulation was abolished by d-tubocurarine, which left the thresold to muscle stimulation unaltered, then addition of SKF 525-A abolished the response to muscle stimulation (Table I).

(d) *Cholinesterase.* SKF 525-A in a concentration of 2 μg/ml caused a 67% inhibition of plasma cholinesterase. In comparison, eserine in a concentration of 1 μg/ml caused complete inhibition.

Discussion. The observations reported here indicate that SKF 525-A has actions in addition to those previously described. SKF 525-A has been regarded as a drug of very low toxicity; the LD_{50} for rats has been reported as 2140 ± 400 mg/kg(2) and for mice approximately 540 mg/kg(3). All the reported actions of SKF 525-A have been concerned with detoxifying enzymes and its effect on them. In such *in vivo* experiments no side effects have been reported.

TABLE I. Effect of Saline (0.9% NaCl), d-Tubocurarine (1 μg/ml), and SKF 525-A (100 μg/ml) on Electrical Stimulation of Frog Gastrocnemius Muscle.

		Threshold for	
Time (min.)	Solution	Nervous stimulation (volts)	Muscle stimulation (volts)
0	.1 ml saline	.065	.32
5	*Idem*	.072	.27
10	"	.070	.29
15	"	.071	.26
20	.1 ml d-tubocurarine	.076	.27
25	*Idem*	.080	.30
30	"	.085	.25
35	"	.110	.27
40	"	34	.28
45	"	>100	.25
50	.1 ml d-tubocurarine + .1 ml SKF 525-A	"	.34
55	*Idem*	"	.38
60	"	"	.50
65	"	"	10
70	"	"	>100
75	"	"	"
80	"	"	"
85	"	"	"
90	"	"	"

The mechanism of the actions of SKF 525-A reported here is difficult to conceive. The most striking and consistent effect was an inhibition of muscle responses to acetylcholine. On the frog rectus muscle SKF 525-A would seem to act by combining with some ACh-sensitive receptor. The initial increase in tension could then be attributed to activation of the receptor site as the drug combined with it. This is similar to the explanation for the acetylcholine-like effect of atropine on the Mytilus gill cilia put forward by Bülbring, Burn, and Shelley(4). The binding appeared to be of a very tenacious nature since the muscle remained insensitive to ACh even after repeated washings with frog Ringer's solution.

The action of SKF 525-A on the rabbit ileum was unusual in that spontaneous rhythmic activity remained after addition of the drug, although the preparation was now insensitive to ACh. With atropine both the spontaneous waves and the response to ACh were abolished. The ileum differed from the frog rectus abdominis muscle in that inhibition of the ACh response could be reversed by frequent washings with fresh Tyrode's solution.

The experiments on stimulation of the frog gastrocnemius muscle indicated that SKF 525-A acted directly on the muscle and not exclusively on the myoneural junction. The frog gastrocnemius muscle was highly sensitive to SKF 525-A, and doses which completely inhibited the response to electrical stimulation had little effect on the responses to electrical stimulation of the other muscles of the same leg.

The inhibition of pseudocholinesterase may be of significance in that no physiological role has been assigned to this enzyme. Its concentration in rat liver is very high, and it may be that it is in some way linked with the microsomal enzymes. The non-specific effects of SKF 525-A might thus be mediated through inhibition of pseudocholinesterase. This would explain how SKF 525-A can inhibit so many diverse systems by a single mechanism. An interesting corollary to this suggestion is a recent observation (Milton, unpublished) that physostigmine a well known pseudocholinesterase inhibitor potentiates barbiturate sleeping time, a prominent property of SKF 525-A.

The significance of the results reported here is difficult to assess in absolute terms, but these data indicate that SKF 525-A exhibits pharmacological properties which are not related to its well known inhibition of detoxifying enzymes.

Summary. 1. SKF 525-A caused an increased tension in the isolated frog rectus abdominis muscle. After treatment with SKF 525-A the response to acetylcholine (ACh) was inhibited, and the inhibition could not be reversed by repeating washing of the muscle with fresh Ringer's solution. The inhibition differed from that observed with d-tubocurarine and atropine. 2. SKF 525-A had no direct effect in low concentrations on the isolated rabbit ileum, but it prevented spontaneous rhythmic activity in higher concentrations. It inhibited the effect of ACh on the gut and this inhibition was reversed by repeated washings with fresh Tyrode's solution. 3. SKF 525-A raised the threshold to direct electrical stimulation of the frog gastrocnemius muscle. 4. SKF 525-A inhibited the enzyme pseudocholinesterase. 5. These data are not fully explained, but the results indicate that SKF 525-A has properties which differ markedly from those previously reported concerning the inhibition of microsomal detoxifying enzymes.

We wish to express our thanks to Dr. R. E. Gosselin for the privilege of working in his laboratory. Smith, Kline, and French kindly supplied the samples of SKF 525-A.

1. Brodie, B. B., *J. Pharm. & Pharmacol.*, 1956, v8, 1.

2. Cook, L., Toner, J. J., Fellows, E. J., *Fed. Proc.*, 1953, v12, 313.

3. Toner, J. J., Cook, L., Fellows, E. J., *ibid.*, 1953, v12, 373.

4. Bülbring, E., Burn, J. H., Shelley, H. J., *Proc. Roy. Soc.* (London) B, 1953, v141, 445.

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Effect of Nicotinic Acid and Related Compounds on Incorporation of Mevalonic Acid into Cholesterol. (26565)

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The hypocholesterolemic effect of nicotinic acid administered orally to humans was first reported by Altschul *et al.*(1). Their results were confirmed by others(2,3). Nicotinic acid was later shown to lower serum cholesterol levels in cholesterol-fed rabbits(4), and a similar effect was observed in dogs(5). The mechanism by which nicotinic acid exerts the hypocholesterolemic effect has not been elucidated. Merrill(6) and later Hardy *et al.*(7) reported an increased incorporation of acetate-1-C^{14} into liver sterols in the presence of nicotinic acid, both *in vitro* and *in vivo*. However, Perry(8) found a decreased incorporation of acetate-2-C^{14} into cholesterol in rat liver slices incubated with nicotinic acid. Kritchevsky *et al.*(11) showed that nicotinic acid-treated rat liver mitochondrial preparations oxidize cholesterol to a greater extent than do similar preparations from control rats.

In the present experiments the effect of nicotinic acid and related compounds on incorporation of mevalonic acid-2-C^{14} (MVA-2-C^{14}) into non-saponifiable material (NSF, largely sterols) by rat liver homogenates was investigated. The afore-mentioned authors (6,7,8) had employed rat liver slices, while in the present studies rat liver homogenates were used. To permit comparison of our data with those of the other authors(6,7,8), the influence of nicotinic acid on conversion of acetate-1-C^{14} to sterols was tested in our system.

Methods and materials. The experimental procedure used to measure incorporation of MVA-2-C^{14} into sterols has been reported (9). Rat liver homogenates were prepared as described previously(9). Female and male rats weighing 200-360 g were employed. MVA-2-C^{14} (New England Nuclear Corp.) was isolated from the dibenzylethylenediamine salt (DBED); 0.5 mg MVA (0.05 μc) was added per flask. Where acetate was used, each flask contained 0.8 mg sodium acetate (1 μc). Nicotinic acid (General Biochemical Co.), isonicotinic acid, isonicotinic acid hydrazide, kynurenic acid, trigonelline, 6-OH nicotinic acid, and pyridine-3-sulfonic acid (the latter 6 compounds were obtained from Nutritional Biochemical Co.) were tested at levels of 1 mg/ml to 5 mg/ml. The excess acidity of these compounds was neutralized with KOH. Each flask contained 5 ml of rat liver homogenate, 1 mg ATP, 10 mg DPN, and 162 mg sodium succinate. Total volume per flask was 10 ml. Ribonuclease (RNAse), Worthington, 5 or 10 mg per flask and $CaCl_2 \cdot 2H_2O$ (7.5 mg per flask), where indicated, were used as "negative controls". Both of these materials have been shown previously to inhibit conversion of MVA to sterols(12,13). Homogenization and additions of ATP, DPN, and RNAse were at 4°C. The flasks were prepared by adding the homoge-

TABLE I. Effect of Various Compounds on Biosynthesis of NSF from MVA.

Exp.	Supplement	Amount, mg	NSF, cpm
1	None	—	4,370
	Nicotinic acid	1	4,259
	Idem	2	4,763
	"	5	4,557
	"	10	3,886
	"	20	3,914
	"	50	3,596
	Isonicotinic acid hydrazide	1	4,323
	Idem	2	4,326
	"	5	4,388
	"	10	5,533
	"	20	5,133
	"	50	6,109
	Pyridine-3-sulfonic acid	1	4,394
	Idem	10	4,233
	"	50	3,288
	Calcium chloride	7.5	3
2*	None	—	6,450
	Nicotinic acid	2	6,344
	Idem	10	6,290
	"	50	6,102
	Isonicotinic acid	1	6,284
	Idem	2	6,265
	"	5	6,347
	"	10	6,223
	"	20	6,179
	"	50	5,633
	Trigonelline	1	6,125
	"	10	6,321
	"	50	6,823
	6-OH Nicotinic acid	1	6,189
	Idem	10	6,177
	"	50	6,352
	Calcium chloride	7.5	0
3	None	—	4,394
	Nicotinic acid	2	4,115
	Idem	10	3,844
	"	50	2,973
	Kynurenic acid	2	4,572
	Idem	10	4,377
	"	50	2,519
	RNAse	5	181
	"	10	137

* No preincubation — substrate and compound tested were both added before incubation. Preincubation in all other experiments.

nate and above indicated constituents. They were aerated with O_2, stoppered tightly, and incubated at 37°C in a shaker bath (50 oscillations per min). Preincubation was for 30 min, after which the substrate was added and the aeration repeated. The flasks were then reincubated for 3 hours. When no preincubation was employed the flasks were incubated for 3½ hours. The reaction was stopped by addition of 10 ml of alcoholic KOH, and the mixtures saponified on a steam bath overnight. The NSF was extracted, and counted by means of a Packard Tricarb-Scintillation Counter as reported previously(10). The data are recorded as counts per min per flask.

Results. Nicotinic acid, isonicotinic acid, isonicotinic acid hydrazide, pyridine-3-sulfonic acid, trigonelline, and 6-OH nicotinic acid each had no significant effect on incorporation of MVA to NSF (Table I). At the highest level tested, 50 mg/flask in one experiment, a small decrease (about 25%) in incorporation of MVA was observed with nicotinic acid and pyridine-3-sulfonic acids. This effect is not considered significant since the decrease occurred only at this high level; moreover, in another experiment the same level of nicotinic acid had no effect (Table I).

Isonicotinic acid hydrazide seemed to cause a slight increase in MVA conversion to NSF at the higher levels (Table I). Kynurenic acid had no effect, except at the highest level, 50 mg/per flask in which case there was a decrease (Table I). RNAse and $CaCl_2$ were each tested in representative experiments and inhibition of MVA incorporation into NSF occurred with each compound (Table I). The data in Table II represent the averaged results of experiments with acetate-1-C^{14}. Considerable variation occurred in the observed values, hence the data were averaged. A 34% average decrease in acetate incorporation was observed at the 10 mg level of nicotinic acid. This finding is in agreement with that of Perry(8) who obtained approximately 25% reduction with the same level of nicotinic acid.

Discussion. Since it had been reported

TABLE II. Effect of Nicotinic Acid on Biosynthesis of NSF from Acetate-1-C^{14}.

Supplement	Amount, mg	NSF, cpm	Decrease in biosynthesis, %
Nicotinic acid	0	1032	0
Idem	1	1104	0
"	10	684	34
"	20	464	55
"	50	351	66

that nicotinic acid influences incorporation of acetate in sterols, it seemed desirable to determine whether or not nicotinic acid has any effect on conversion of MVA to NSF. The data indicate that nicotinic acid does not influence formation of sterols from MVA. Several nicotinic acid-related compounds likewise have little or no influence on MVA conversion to NSF. The previous experimenters had employed rat liver slices to investigate acetate incorporation into sterols. In the present experiments rat liver homogenates were used. Therefore the effect of nicotinic acid on acetate-1-C^{14} conversion to NSF was reinvestigated with homogenates. Considerable variation in ability of control homogenates to convert acetate to NSF was observed, in contrast to their ability to convert MVA to NSF, and consequently the data (Table II) were averaged. The resulting data indicated a decrease in amount of acetate converted to NSF as the concentration of nicotinic acid increased. These findings are in agreement with those of Perry who found a decrease in conversion of acetate (approx. 25%) to cholesterol using 1 mg per ml of nicotinic acid. Hardy *et al.* have reported an increase in acetate incorporation into sterols using 0.05 M nicotinic acid (6.15 mg/ml) but a decrease at 0.10 to 0.25 M (about 12 to 30 mg/ml) nicotinic acid. The present data indicate that the locus of the nicotinic acid effect on cholesterol metabolism, at least in homogenates, is before MVA or at some other position which indirectly influences cholesterol metabolism. The findings with acetate suggest that the effect takes place somewhere between acetate and MVA, since nicotinic acid inhibited acetate incorporation into NSF but does not inhibit MVA incorporation into NSF.

Summary. Nicotinic acid and related compounds (0.1 to 5 mg per ml) have no significant effect on conversion of mevalonic acid to non-saponifiable material by rat liver homogenates. The incorporation of acetate into non-saponifiable material (largely cholesterol) decreases with increasing amounts of nicotinic acid. At 1 mg per ml of nicotinic acid a 34% average decrease in biosynthesis was observed. The results suggest that the locus of the nicotinic acid effect may be somewhere between acetate and mevalonate.

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1. Altschul, R., Hoffer, A., Stephen, J. D., *Arch. Biochem.*, 1955, v54, 558.
2. Parsons, W. B., Flinn, J. H., *Circulation,* 1957, v16, 499.
3. Achor, R. W., Berge, K., Barker, R. N. W., McKenzie, B. F., *ibid.*, 1958, v17, 497.
4. Merrill, J. M., Lemely-Stone, J., *Circulation Res.*, 1957, v5, 617.
5. Nava, A. F. C., Lozano, E., Fishleder, B. L., Sodi-Pallares, D., *Am. Heart J.*, 1958, v56, 598.
6. Merrill, J. M., *Circulation Res.*, 1958, v6, 482.
7. Hardy, R. W. F., Gaylor, J. L., Baumann, C. A., *J. Nutrition,* 1960, v71, 159.
8. Perry, W. F., *Metabolism,* 1960, v9, 686.
9. Wright, L. D., Cleland, M., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 219.
10. Wright, L. D., Loeb, M., Hanson, R., *ibid.*, 1960, v103, 546.
11. Kritchevsky, D., Whitehouse, M. W., Staple, E., *J. Lipid Res.*, 1960, v1, 154.
12. Wright, L. D., Cleland, M., Dutta, B. N., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 425.
13. Wright, L. D., Li, L. F., Unpublished results.

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Ascites Production in 17 Mouse Strains. (26566)

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Ascitic fluid from immunized mice as a source of high titer antibody has been reported(1-4). Interest in ascites production arises also from the fact that with the BALB/cAnN mouse, ascites may be associated with development of plasma cell tumor(5).

Success in eliciting ascites through use of incomplete Freund's adjuvant has not been uniform in this laboratory. Judging from numerous personal communications from other investigators, difficulties have also been encountered elsewhere. The present report is concerned with factors affecting the reproducibility of the phenomenon of ascites production.

The principal factor reported here is that of mouse strain variation in ascitic fluid production and, in some instances, the effect thereon of including heat-killed *Staphylococcus aureus* in the ascites-inducing dose. Variation in the time course of production of ascitic fluid and the lethality of treatment are also observed . For the BALB/cAnN mouse, the occurrence of plasma cell tumors is noted.

Materials and methods. Procedures employed in the various experiments covered in this report have been described(4). Female mice of 17 different strains, weighing approximately 20 g each, were housed in groups of 5 per strain. Mice of the same strains, where indicated, were preimmunized with horse serum. Two injections of mixtures of 0.1 ml horse serum and 0.1 ml incomplete Freund's adjuvant were injected subcutaneously at 2 week intervals to each mouse. Three weeks following the first preimmunizing dose, the ascites inducing regimen was initiated.

All mice, except where otherwise indicated, received 0.5 ml intraperitoneal inoculations of equal parts of incomplete Freund's adjuvant and heat-killed 10^9 *Staphylococcus aureus* #18 cells in trypticase soy broth. Injections were administered 3-5 times at intervals of 3-5 days. Those mice receiving adjuvant alone were treated in a similar manner except that physiologic saline was substituted for the staphylococcus. In isolated instances similar booster inoculations were given 3-6 months following primary inoculation.

No prescribed schedule was followed for removal of the ascitic fluid. Mice were "tapped" at the convenience of the laboratory when their appearance indicated ascites.

Results. Fig. 1 shows results of separate experiments for 5 mouse strains and the major details of immunization and of treatment with incomplete Freund's adjuvant or *S. aureus*-incomplete Freund's adjuvant mixtures. The data shown are cumulative average yields of ascitic fluid and times of death of individual mice. The extent to which early death is responsible for poor ascites production can be judged from the data.

Striking strain differences in patterns and levels can be seen. Characteristically, CDF_1 yields are low while those for AL/N are high. With the use of *S. aureus*, good yields of ascitic fluid usually occur earlier. With adjuvant only, production is delayed for about 6 months but subsequent production can be extremely high, more than compensating for the low early yield.

The CAF_1 mouse shows reasonably high production in 2 of 3 experiments where *S. aureus* was employed. Without *S. aureus* production was delayed and remained low over the 10 month observation period.

BALB/cAnN production employing *S. aureus* was low, partially due to early fatality occurring in this strain. With adjuvant alone, however, delayed production attained high levels. These increased yields were associated with plasma cell tumors,* occurring in

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* We are grateful to Dr. John H. Edgcomb, Pathologic Anatomy Dept., Nat. Cancer Inst., for pathologic examinations of the mice.

TABLE I. Strain Variation in Production of Ascites in Mice.

				Ascitic fluid produced, ml					20% best producers of mice with ascites		% survival		
					Avg per mouse								
						Mo					Mo		
Strain	Total No. mice used	No. exp. done	% ascites	Total in period	Entire period	1–3	4–6	7–9	Yield range, ml	% of total yield	3	6	9
Inbred strains				*9 month studies*									
BALB/cAnN	83	6	51	671	8.1	2.2	.6	5.3	31– 77	55	60	48	30
DBA/2JN	63	5	63	536	8.5	2.1	1.6	4.8	20– 92	67	87	78	64
AL/N	35	3	92	525	15.0	6.4	3.1	5.5	29– 61	54	80	54	43
C3H/HeN	7	1	86	130	18.6	2.5	10.6	5.5	38– 82	69	100	100	86
Hybrid strains													
CDF$_1$	35	3	46	62	1.8	.8	.8	.2	6– 17	66	66	57	51
CAF$_1$	63	5	55	359	5.7	4.2	1.0	.5	18– 29	45	81	76	62
AAF$_1$	6	1	100	103	17.1	11.5	5.6	.0	35– 35	41	33	17	17
Inbred strains				*6 month studies*									
C57BL/10ScBs	18	2	89	342	19.0	13.2	5.8	—	26–127	74	56	50	—
AKR/LwN	10	1	50	88	8.8	2.3	6.5	—	34	39	50	10	—
C3H$_f$B/HeN	16	2	50	229	14.3	2.6	11.7	—	43– 99	55	88	69	—
BRSUNT/N	16	2	25	24	1.5	1.0	.5	—	16	64	62	44	—
C57BL/10-H-2^{d}N	10	1	0	0	.0	.0	.0	—	—	—	100	60	—
STR/N	10	1	0	0	.0	.0	.0	—	—	—	100	100	—
C57L/HeN	9	1	78	109	12.1	1.4	10.7	—	22– 53	57	100	100	—
Hybrid strains													
LAF$_1$	16	2	38	42	2.6	.1	2.5	—	8– 30	76	75	69	—
CF$_1$	14	1	86	83	5.9	4.2	1.7	—	7– 55	80	86	86	—
CAF$_2$*	16	1	50	16	1.0	1.0	.0	—	4– 7	61	62	62	—

* CAF$_2$ derived from brother-sister matings of mice in a single CAF$_1$ litter.

10 of the 19 mice surviving beyond 6 months.

The pattern of ascitic fluid yield for the DBA/2JN mouse parallels somewhat that for the BALB/cAnN in each of the experiments. In the former strain however, mortality is less and no plasma cell tumors occurred. The pattern of earlier production of ascitic fluid employing *S. aureus* is also observed for both the strains.

Table I gives a summary of results for 17 mouse strains. The combined data shown cover several experiments for most strains. All groups except those already indicated in Fig. 1 received *S. aureus*-incomplete Freund's adjuvant mixtures.

The data shown, which cover either a 6 or 9 month period, include percentage of ascitic mice, yield per mouse in each 3 months period, and percentage of mice surviving each such period. Production by individual mice was sometimes very large: one C57BL/10 ScBs mouse produced 127 ml; a BALB/cAnN mouse, with a plasma cell tumor which produced 23 ml in the 9 month period, went on to produce 181 ml before it was sacrificed in the 11th month. Good average yields were not generally due solely to such exceptional producers; other mice in the same treatment group generally showed moderately high yields. For those mice developing ascites, the table shows the range of production by the 20% best producers and what percentage of the total yield, typically about 60%, came from these.

Discussion. Both total ascitic fluid yield and its course of production must be considered in planning use of such fluid as a source of potent antibody. The need for early production may outweigh the desirability of greater but delayed yields. Under study is the question whether increased production of fluid occurs without loss of titer or merely represents dilution.

Mice with minimal ascites could have been overlooked because they were "tapped" when their appearance indicated ascites, rather than at specified times. Also, mortality might have been less had the mice been "tapped"

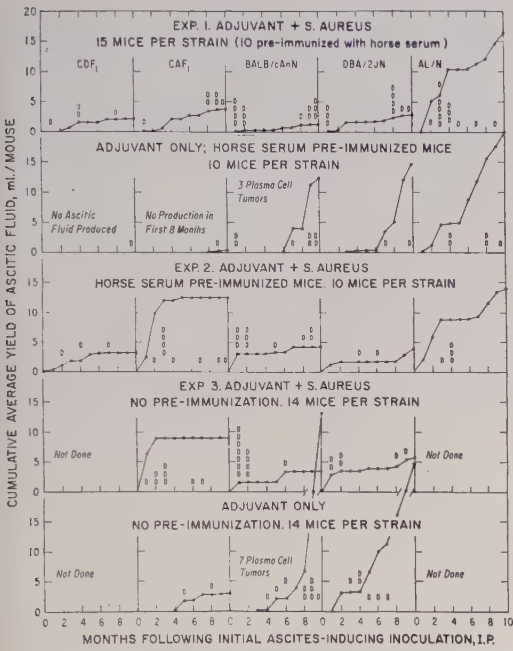

FIG. 1. Ascitic fluid production following inoculation with incomplete Freund's adjuvant alone or with heat-killed *S. aureus*. Cumulative average yields shown are based on total number of mice in experiment. Deaths of individual mice are indicated by letter *D*.

sooner and undue accumulation of fluid avoided. These alterations in technic might have led to higher incidence rates and perhaps also to higher yields than were observed.

Plasma cells have been implicated in antibody production(6) and if the property also resides in the plasma cell tumor, its occurrence in the BALB/cAnN mouse may provide a useful tool. A strain-specific carcinogenic role for the components of the adjuvant employed is suggested by the results for these mice.

Summary. Mouse strains vary in their production of ascitic fluid. Intraperitoneal injections of heat-killed *Staphylococcus aureus*-incomplete Freund's adjuvant mixtures induce early development of ascites. Yield of ascitic fluid may be greater with adjuvant alone but production is often delayed. Yield in individual mice may be very large. High late yield for BALB/cAnN mice receiving only adjuvant are associated with occurrence of plasma cell tumors.

1. Munoz, J., PROC. SOC. EXP. BIOL. AND MED., 1958, v95, 757.
2. Lieberman, R., Douglas, J. O. A., Humphrey, Wm., Jr., *Science*, 1959, v129, 775.
3. Kasel, J., Lieberman, R., Smith H. A., *Virology*, 1959, v9, 702.
4. Lieberman, R., Douglas, J. O. A., Mantel, N., *J. Immunol.*, 1960, v84, 514.
5. Potter, M., Robertson, C. L., *J. Nat. Cancer Inst.*, 1960, v25, 847.
6. Fagraeus, A., *J. Immunol.*, 1948, v58, 1.

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Red Cell Factor in Renal Damage from Hypertonic Solutions.* (26567)

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Previous work has demonstrated that the intravascular administration of certain solutions having a concentration greater than 1500 mOsm/L will produce red cell agglutination (1). This effect has been demonstrated in the lung, mesentery, brain and extremities of both cats and dogs(2). Removal of the red cell from the circulation to these areas abolishes the increase in vascular resistance which usually follows injection of markedly hypertonic media. Since the phenomenon of intravascular aggregation has been shown to be a cause of death in cardioangiography(3) and the mechanism responsible for ischemic arteriographic complications(4), we have been interested in determining its possible role in renal damage following aortography. As a first step, the following study was undertaken to delineate those changes in the kidney which result from osmotic activity alone.

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Methods. Adult mongrel dogs, 8 to 12 kg in weight, were anesthetised with 30 mg/kg of pentobarbital sodium. The aorta above and below the renal arteries was exposed through a midline abdominal incision. Vascular clamps were placed proximal and distal to this segment and upon the right renal artery, thus enabling that kidney to serve as a control. The left kidney was immediately perfused from a reservoir at a pressure of 150-200 mm Hg, through a needle inserted into the aortic segment. Flow rates were in the range of 30-60 ml/min. The perfusate was either autologous heparinized blood or plasma; the latter obtained 10 days previously by phlebotomy. One ml/kg of either 12.5% sodium chloride or 25% urea in 0.9% saline was injected rapidly 30 to 60 seconds after onset of perfusion. Total time to perfusion was 2 minutes or less in all cases. Vascular clamps were then removed and normal circulation restored to both kidneys. The aorta at point of injection was repaired with fine arterial silk, taking care that no constriction resulted. The animals were maintained for a period of 7 days under standard kennel conditions. Intravenous fluids were administered as necessary and each dog received antibiotics for 4 days following the procedure. On the seventh day they were sacrificed following intravenous pyelography, and the kidneys removed for gross and microscopic study.

A subsequent group was operated upon with one modification. The right ureter was ligated prior to perfusion so that the injected side would remain as the sole functioning renal tissue. Sodium chloride (12.5%) was injected as before during blood and plasma perfusion. Daily blood urea nitrogen determinations were made over the next 7 days followed by sacrifice.

Results. A total of 36 animals were used in the experiment. Twelve received urea injections in the presence of either blood or plasma, and the remaining 24 were injected with 12.5% sodium chloride under the same conditions. Results in the injected kidneys have been divided into 2 groups depending upon which solution was used. In each instance the kidney which served as a control and was therefore not exposed to hyperosmotic materials concentrated well on intravenous pyelography, and was normal at postmortem examination.

Kidneys injected with 25% urea in 0.9% saline. Injections of 25% urea in 0.9% saline into both blood and plasma perfused kidneys failed to produce significant renal damage. All 12 animals were clinically well during the course of the experiment. Good visualization was obtained on intravenous pyelography and at autopsy. The only pathology noted was an occasional area of tubular vacuolization.

Kidneys injected with 12.5% sodium chloride. In those 12 animals whose kidneys were perfused with plasma, the results were comparable to those previously described for urea. However when blood was used, the dogs were listless, refused to eat and required parenteral fluid support. In no case was the injected kidney seen on intravenous pyelography. At autopsy, diffuse parenchymal hemorrhage with areas of infarction was apparent grossly, and severe renal damage had occurred microscopically (Fig. 1).

The function of kidneys injected with 12.5% sodium chloride was further studied in the modified group of 12 animals. All animals perfused with blood rapidly became uremic. Two died at 72 hr (see Fig. 3). The remaining 4 animals were toxic and exhibited no evidence of improvement to time of sacrifice. Microscopic and gross examination of these organs demonstrated damage similar to that previously described. However mean blood urea nitrogen values for the plasma perfused organs subjected to 12.5% sodium chloride did not rise above 40 mg %; no deaths occurred in this group and their postoperative course was benign.

Discussion. The results show clearly that hypertonic sodium chloride is more damaging to the kidney than an equally concentrated solution of urea. Further this damage can be significantly reduced by removing red cells from the renal circulation. These findings indicate that the renal toxicity of concentrated solutions is dependent to a considerable degree upon the presence of the red cell, rather than being primarily a direct effect upon the kidney substance. This conclusion is in di-

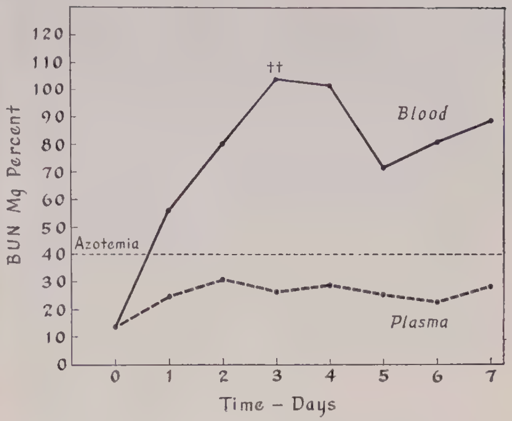
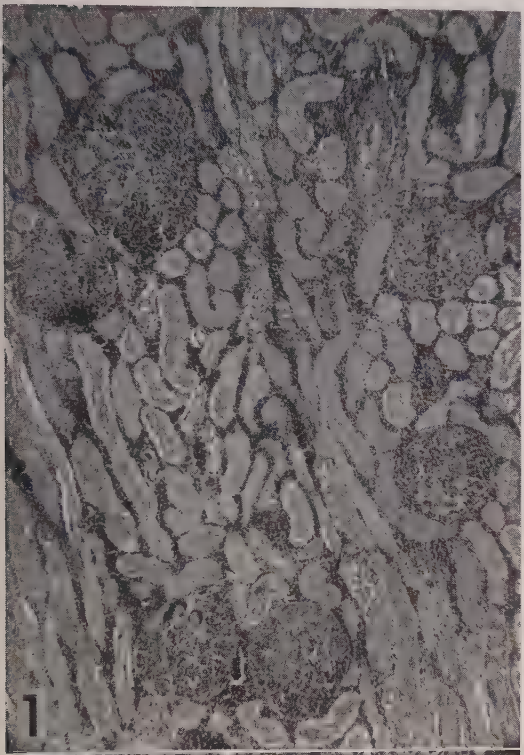


FIG. 3. Mean blood urea nitrogen values in animals injected with 12.5% sodium chloride during blood and plasma perfusion of their sole functioning kidney. Crosses mark the death of 2 animals at 72 hr.

rect opposition to recent work performed with concentrated organic iodide solutions; however the conclusions drawn from this latter investigation were based upon indirect evidence alone(5). It should be realized that the severe changes under consideration are superimposed upon certain microscopic alterations previously categorized as osmotic nephrosis(6). Tubular damage of this latter nature has been described after administration of a number of mildly hyperosmotic materials.

It is interesting to speculate upon the reason for the role of the red cell in the pathogenesis of renal damage from hyperosmotic solutions. The fact that concentrated urea as opposed to isosmolar sodium chloride did not cause necrosis is obviously important. This suggests, in view of the marked difference which exists in their ability to exert an osmotic differential across the red cell membrane, that osmotic damage to the red cell is the significant factor involved. Previous work in other areas of the body has demonstrated



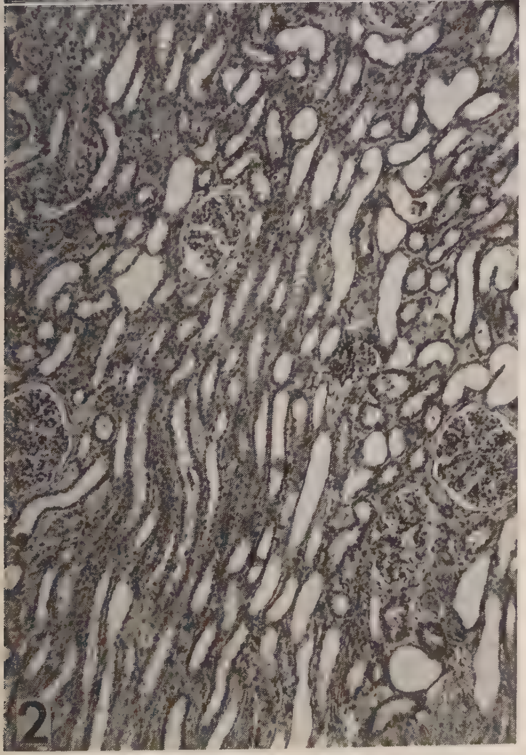


FIG. 1. Renal changes present at 7 days in canine kidney subjected to 12.5% sodium chloride during blood perfusion. All renal elements are involved in the destructive process, which is in essence, an infarct.

FIG. 2. Renal changes accompanying administration of 12.5% sodium chloride during plasma perfusion 7 days following injection. Note retention of architecture, and tubular vacuolization typical of osmotic nephrosis. More severe changes consisting of tubular desquamation and cast formation are present in many areas.

that red cells subjected to a marked osmotic gradient agglutinate and thus produce obstruction to blood flow by a process of microthromboembolism. The present findings are consistent with the hypothesis that this phenomenon is responsible for renal destruction arising from concentrated solutions.

Summary. Laparotomy was performed on 36 mongrel dogs. The aorticorenal segment was isolated and perfused with either blood or plasma. One ml/kg of 12.5% sodium chloride or 25% urea in 0.9% saline was injected during the perfusion. Concentrated sodium chloride produced extensive damage in the blood perfused organ but not when plasma was used. Kidneys injected with 25% urea in 0.9% saline regardless of perfusate did not differ from control organs or from each other. Animals whose only functioning kidneys were injected with sodium chloride developed uremia if blood was used for perfusion. It is concluded that the renal toxicity of concentrated solutions is dependent to a considerable degree upon the presence of the red cell and is not therefore a primary effect upon the renal parenchyma. The importance of the red cell under these circumstances may be related to the phenomenon of intravascular agglutination which has been described previously in other areas of the body following administration of markedly hyperosmotic solutions.

1. Read, R. C., Vick, J., Meyer, M., *Fed. Proc.*, 1959, v18, 124.
2. Read, R. C., Johnson, J. A., Vick, J. A., Meyer, M. W., *Circ. Res.*, 1960, May 8, 538.
3. Read, R. C., *J. Thor. Cardiov. Surg.*, 1959, v38, 685.
4. Read, R. C., Meyer, M., *Surg. Forum*, 1960, v10, 472.
5. Killian, D. A., Owens, G., *Ann. Surg.*, 1960, v152, 957.
6. Allen, Arthur C., *The Kidney*, Grune and Stratton, N. Y., 1951.

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Mitochondrial Swelling Produced by Compounds Exhibiting Estrogenic Activity.* (26568)

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The observation that the synthetic estrogen, diethylstilbesterol, when given together with carcinostatic levels of the niacin antagonist, 6-aminonicotinamide, produces regression in the 755 tumor grown in C_{57} black mice(1) coupled with the demonstration that diethylstilbesterol greatly increases the ability of 6-aminonicotinamide to antagonize various pyridine nucleotide-dependent multienzyme systems(1,2) led us to determine the effect of diethylstilbesterol and other synthetic or natural phenolic compounds possessing estrogenic activity on mitochondrial volume or structure. The report of these findings is the purpose of this communication.

Materials and methods. Mitochondria were isolated from the livers of non-fasted male Holzman rats weighing 250-350 g. Preparation of the mitochondria and the technic employed in measuring mitochondria swelling were essentially that of Tapley(3) except that a Beckman DU spectrophotometer was employed. Inhibitory compounds of reagent grade were dissolved in 95% ethanol immediately before use and were shielded from sunlight. Ethylenediaminetetraacetic acid (EDTA), Mg^{++} and adenosine triphosphate(ATP) were dissolved in the concentrations indicated and adjusted to pH 7.4 before use. Three times glass-distilled water was used throughout.

Results. The stability of rat liver mitochondrial preparations in various concentrations of sucrose with and without diethylstilbesterol is presented in Table I. The results

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TABLE I. Effect of Diethylstilbesterol on Mitochondrial Swelling in Various Concentrations of Sucrose.*

Medium addition	Swelling, △ O.D. × 10^3 at 520 mμ △ 10′	△ 20′	△ 30′
.5 M sucrose	−2	1	0
Idem + diethylstilbesterol	170	220	265
.3 M sucrose	15	35	85
Idem + diethylstilbesterol	160	225	275
.05 M sucrose	270	290	300
Idem + diethylstilbesterol	250	270	275

* Diethylstilbesterol was dissolved in 95% EtOH, so that 0.1 ml when diluted to a total volume of 3.0 ml gave a final molarity of 8.0×10^{-5}M. An equal volume of 95% EtOH was added to the control reactions.

pertaining to mitochondrial stability in sucrose solutions of various strengths are in agreement with those found by others(3,4,5). Mitochondrial preparations were unaffected by 0.5M sucrose. However, in a medium consisting of 0.3M sucrose slow but steady swelling of the mitochondria occurred and in 0.05M sucrose a very rapid swelling was observed. Diethylstilbesterol at a final concentration of 8.0×10^{-5}M accelerates swelling in presence of 0.3M sucrose, but has no effect on the rapid swelling which results when the mitochondria are suspended in 0.5M sucrose. The effect on the swelling of rat liver mitochondria by various compounds with estrogenic activity is presented in Table II.

TABLE II. Effect of Various Estrogenic Compounds on Volume of Rat Liver Mitochondria.*

Medium addition	Swelling, △ O.D. × 10^3 at 520 mμ △ 10′	△ 20′	△ 30′
None	2	7	2
EtOH	1	3	6
Estradiol (8.0×10^{-5}M)	2	3	4
Phloretin (8.0×10^{-5}M)	1	2	5
Diethylstilbesterol			
(4.0×10^{-5}M)	30	62	82
(8.0×10^{-5}M)	160	220	270
Hexosterol (4.0×10^{-5}M)	32	62	84
" (8.0×10^{-5}M)	158	216	268
Dienesterol (4.0×10^{-5}M)	45	80	110
" (8.0×10^{-5}M)	165	230	290

* Mitochondria were suspended in 0.5 M sucrose. All test compounds were dissolved in 95% EtOH, so that 0.1 ml, in a total volume of 3 ml, gave the final molarities indicated.

Estradiol and phloretin at concentrations as high as 8.0×10^{-5}M had no apparent effect on mitochondrial volume. It was impossible to determine accurately the effect of higher concentrations of these compounds owing to their insolubility in an aqueous medium. Diethylstilbesterol, hexosterol and dienesterol at concentrations of 4.0×10^{-5}M produced significant increases in the volume of rat liver mitochondria. Increasing the levels of these 3 synthetic estrogens to 8.0×10^{-5}M markedly increased rate of swelling. No significant difference in the relative ability of either hexosterol, diethylstilbesterol or dienesterol to cause mitochondria swelling was observed. Neither magnesium, ATP (Table III) nor EDTA had an effect on the ability of diethylstilbesterol to produce mitochondria swelling.

TABLE III. Effect of ATP, Magnesium and Versene on Rat Liver Mitochondrial Volumes Changes Produced by Diethylstilbesterol.*

Medium addition	Swelling, △ O.D. × 10^3 at 520 mμ △ 10′	△ 20′	△ 30′
EtOH control	−2	1	2
Diethylstilbesterol	169	217	263
Mg^{++}	0	1	3
" + diethylstilbesterol	168	215	260
ATP	1	1	2
" + diethylstilbesterol	169	216	260
Versene	0	1	6
" + diethylstilbesterol	169	217	262

* Mg^{++} as $MgCl_2$, disodium salt of ATP, and EDTA (1.0×10^{-3}M) final concentration were dissolved in 0.5 M sucrose and neutralized to pH 7.4 before addition to the reaction vessels. Mitochondria were suspended in 0.5 M sucrose. Diethylstilbesterol was added in 95% EtOH to a final molarity of 8.0×10^{-5}M. An equivalent amount of EtOH (0.1 ml) was added to all other reaction vessels.

Discussion. These studies employing liver confirm the observations of Packer and Bacila(6) that diethylstilbesterol causes swelling of heart sarcosomes. The effect of diethylstilbesterol, dienesterol and hexosterol on the volume of hepatic mitochondria differs a bit from the mitochondrial volume changes produced by desoxycortisone, progesterone and other natural steroids on liver and tumor mitochondria(7). In the presence of progesterone and other natural steroids the mitochondria volume changes can be reversed by magnesium or ATP(7). In our studies magnesium or ATP were without effect in reversing the effects of diethylstilbesterol, dienesterol or hexosterol on mitochondrial swelling. The relatively large quantities of inhibitors needed to produce significant changes in mitochondrial volume together with a lack of correlation of the relative abilities of these estrogenic substances to produce swelling and potentiate 6-aminonicotinamide toxicity on the 755 tumor† apparently discount possible *in vivo* changes in mitochondrial volume, produced by these compounds, as being pertinent to the biological results. Thus, subsequent increases in mitochondrial permeability to 6-aminonicotinamide or pyridine nucleotide analogues containing 6-aminonicotinamide may be ruled out as a major factor in enhancing 6-aminonicotinamide antagonism of intramitochondrial enzymes dependent on pyridine nucleotide.

Summary. The ability of the compounds diethylstilbesterol, hexosterol, dienesterol, phloretin and estradiol to induce swelling of liver mitochondria was determined. Diethylstilbesterol, hexosterol and dienesterol produced marked mitochondrial swelling at final concentration of 4-8 × 10^{-5}M. The compounds estradiol and phloretin were inactive at the highest levels tested (8 × 10^{-5}M). Under the experimental conditions ATP, Mg^{++} or versene did not prevent the mitochondrial swelling induced by diethylstilbesterol.

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† Diethylstilbesterol and hexosterol are far more effective than estradiol, dienesterol and phloretin in potentiating the anti-tumor activity of the niacin antagonist 6-aminonicotinamide in the 755 tumor grown in 755 black mice (unpublished data, Dietrich, L. S. and Martin, D. S.).

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1. Martin, D. S., Dietrich, L. S., Fugman, R. A., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 58.
2. Dietrich, L. S., Martin, D. S., *Cancer Res.*, 1961, v21, 361.
3. Tapley, D. F., *J. Biol. Chem.*, 1956, v222, 325.
4. Packer, L., *ibid.*, 1960, v235, 242.
5. Raaflaub, J., *J. Helv. Physiol. Acta*, 1953, v11, 142.
6. Packer, L., Bacila, M., *Acta Physiol. Latino Am.* 1958, v8, 230.
7. Blecher, M., White, A., *J. Biol. Chem.*, 1960, v235, 3404.

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Proteins in Normal Cerebrospinal Fluid Not Found in Serum.* (26569)

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Recent reports dealing with proteins in the cerebrospinal fluid (CSF) have neither proved nor disproved the existence of proteins specific for CSF and not found in serum. Paper electrophoresis(1,2,3) does not demonstrate antigenic differences between the 2 fluids. The commercially available antiserum against human serum proteins has been used in several immuno-electrophoretic investigations of CSF (see survey by Burtin(4) and by Clausen (5)). These investigations, however, did not reveal the existence of special CSF proteins not found in serum. Immuno-electrophoresis using specific sera against CSF is a reliable method of demonstrating differences between proteins of CSF and proteins of serum. This communication adds some data to the very few results obtained in this field(6).

Materials and methods. Normal cerebrospinal fluids were obtained from patients who had undergone pneumo-encephalography at The Municipal Hospital, Copenhagen, Denmark. These patients had no other neurological complaints than cephalalgia, without objective findings.

Immuno-electrophoresis was performed as described by Scheidegger(7). The antisera made in this laboratory were manufactured as described previously(8). The following antisera were used: 1) Rabbit anti CSF anti-

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* Supported by the Danish League Against Multiple Sclerosis, Copenhagen, Denmark.

serum (No. 11), made in this laboratory. 2) Rabbit anti human gamma globulin antiserum from Behringwerke Marburg/Lahn, Germany.

The CSF used for immunization and immuno-electrophoresis was pooled from several persons and concentrated to 5 g% (w/v) by suction dialysis in an apparatus and membranes from Membrangesellschaft, Göttingen, Germany.

The 5 CSF gamma globulin fractions, found in agar gel micro-electrophoresis especially distinctly in CSF from patients with infectious diseases, were demonstrated with agar gel micro-electrophoresis of CSF from a patient with lymphocytic meningitis as described by Loewenthal *et al.*(9).

Results. Fig. 1 shows the immuno-electrophoresis of normal CSF developed with specific antiserum No. 11. The findings are correlated to similar tracings obtained when No. 11 developed normal human serum.

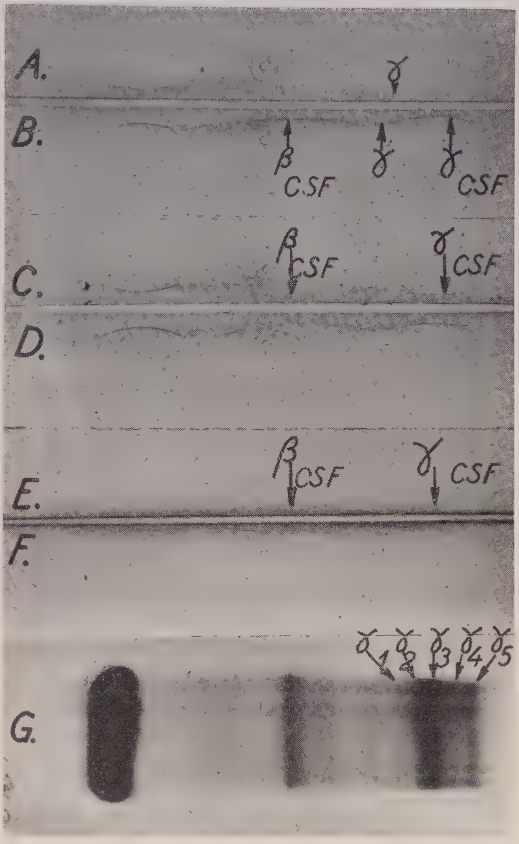

FIG. 1. Immuno-electrophoretic findings in normal cerebrospinal fluid. Demonstration of 2 specific proteins in CSF by means of a specific anti-CSF antiserum (No. 11). A. Imuno-electrophoresis of normal human serum developed with the anti-CSF antiserum No. 11. Only one precipitation line corresponding to the conventional serum gamma globulin in gamma area is seen. B. Normal concentrated CSF developed as A. Beside the serum gamma precipitation line precipitation lines corresponding to beta-CSF and gamma-CSF are visualized. C and D. Same tracings as B. E and F. Same tracings as C and D developed with anti-CSF antiserum absorbed with normal human serum in the proportion 3:1. Precipitation lines corresponding to the 2 specific proteins: beta-CSF and gamma-CSF are seen selectively. G. Agar gel micro-electrophoresis of CSF from a patient with lymphocytic meningitis, demonstrating the 5 gamma globulin fractions, of which some might correspond to the gamma-CSF fraction found in immuno-electrophoresis.

One prealbumin line, one albumin line, and several precipitation lines in the alpha- and beta-areas corresponding to similar lines in human serum immuno-electrophoresis can be seen. These lines are neither visualized in immuno-electrophoresis when antiserum 11 is absorbed with normal human serum nor when CSF is developed with specific anti gamma globulin. Thus all the precipitation bows in the prealbumin, albumin, and alpha-areas correspond to antigenically closely related proteins in human serum. On the other hand, 2 precipitation lines in the beta- and gamma-areas, called beta-CSF and gamma-CSF, can also be developed in immuno-electrophoresis with anti-CSF 11 absorbed with normal human serum. This means that these 2 proteins might be specific for CSF.

The precipitation line corresponding to beta-CSF corresponds to a group of antigenically identical proteins with different mobilities, from the slow albumin zone to the intermediate gamma globulin area.

Gamma-CSF has a small interval of mobility from the intermediate gamma-area to a mobility somewhat slower than the slowest gamma-mobility in serum.

Discussion. Previous investigations seem to indicate differences between CSF and human serum, for example, the tau-fraction found in paper electrophoresis(10), and the 5 gamma globulin fractions in CSF revealed in agar gel micro-electrophoresis(11). These investigations could not, however, show any possible antigenic differences between the proteins of the 2 media. Immuno-electrophoresis has shown that the tau-fraction possibly corresponds to enzymatically changed transferrin, which has preserved the antigenic properties involved in the immuno-electrophoretic procedure(12,13,14).

Chevance, Galli, Jeanmaire, and Gerard(6) have recently described an immuno-electrophoresis with specific CSF antiserum. They have described 9 precipitation lines, among them a slowly moving gamma globulin. However, as mentioned by Burtin, the explanation of the immuno-electrophoretic findings was limited by lack of a potent antiserum. The data given here support the findings by Chevance *et al.*(6) of a slowly moving fraction in CSF with a mobility slower than that of the serum gamma globulin, antigenically different from serum gamma globulin. Both the absorption experiments with normal human serum and the crossing of the 2 slowest moving precipitation lines seem to exclude a very close antigenic relation between serum gamma globulin and gamma-CSF (Fig. 1). These findings may account for some of the slowest moving gamma globulin fractions revealed in agar gel micro-electrophoresis and explain the selective increase of these in diseases of the central nervous system (Fig. 1).

Summary. Immuno-electrophoresis of normal concentrated cerebrospinal fluid, developed with a specific rabbit anti cerebrospinal fluid antiserum, has demonstrated the existence in the gamma and beta areas of 2 proteins not found in serum.

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1. Kabat, E. A., Landow, H., Moore, D. H., PROC. SOC. EXP. BIOL. AND MED., 1942, v49, 260.

2. Kabat, E. A., Moore, D. H., Landow, H., *J. Clin. Invest.*, 1942, v27, 571.

3. Grabar, P., Williams, C. A., *Biochim. biophys. Acta*, 1953, v10, 193.

4. Burtin, P., in *Analyse Immuno-électrophorétique,* Grabar, P., Burtin, P., Ed., Masson et Cie, 1960, 245.

5. Clausen, J., *World Neurol.*, 1960, v6, 479.

6. Chevance, L. G., Galli, A., Jeanmaire, J., Gerard, G., in *Analyse Immuno-électrophorétique,* Grabar, P., Burtin, P., Ed., Masson et Cie, 1960, 253.

7. Scheidegger, J. J., *Internat. Arch. Allergy,* 1955, v7, 103.

8. Heremans, J., Clausen, J., Heremans, M. Th., Rask-Nielsen, R., *J. Nat. Cancer Inst.*, 1959, v22, 45.

9. Loewenthal, A., Karcher, D., Van Sande, M., in Protides of the Biological Fluids, Peters, H., Ed., Elsevier Publ. Comp., 1960, 309.

10. Bücher, Th., Matzelt, D., Pette, D., *Klin. Wschr.*, 1952, v30, 325.

11. Loewenthal, A., Van Sande, M., Karcher, D., *J. Neurochem.*, 1960, v6, 51.

12. Pette, D., Stupp, I., *Klin. Wschr.*, 1960, v38, 109.

13. Clausen, J., *Acta Psych. Scand.*, 1960, v35 (Suppl. 148), 11.

14. Clausen, J., Munkner, T., *Nature,* 1961, v189, 60.

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A Study of the Mechanism of the Increase in Cardiac Output Induced by Isopropylarterenol Hydrochloride (Isuprel®).* (26570)

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Many investigators(1-5) have studied the effect of isopropylarterenol hydrochloride (Isuprel®) on various aspects of the circulation and have demonstrated that the drug increases heart rate and cardiac output, decreases mean arterial blood pressure or leaves it unchanged, and lowers total peripheral resistance. To evaluate the role of the rise in heart rate normally induced by Isuprel in the genesis of the increase in cardiac output caused by the drug, the effect of Isuprel on

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cardiac output and arterial blood pressure of the whole animal was investigated while heart rate was kept constant during the period of action of the drug.

Methods. Ten dogs (18.2-37.7 kg) were anesthetized by intravenous administration of 100 mg of chloralose per kg. The methods used to measure and record continuously the output of the left ventricle (CO), and the mean arterial blood pressure (MABP), as well as the formula used to calculate total peripheral resistance (TPR) have been described(5-10). The heart rate (HR) was obtained from electrocardiographic tracings. Atrial (4 experiments on 2 dogs) or ventricular (13 experiments on 8 dogs) tachycardia of the desired rate was induced electrically with a Grass stimulator, short DC stimuli of minimal intensity being applied to the left atrium or the left ventricle through 2 fishhook electrodes. In a first series of 8 experiments on 5 dogs (Part A, Fig. 1), after a control period, tachycardia was induced, then after 65 to 184 seconds of tachycardia a 0.0005% Isuprel solution in 0.9% NaCl solution was administered intravenously at the average rate of 4.6 ml/min, while tachycardia was maintained. Administration of the drug lasted from 100 to 176 seconds. Amount of the drug administered ranged between 0.9 and 3.6 μg/kg. Administration of the drug was then discontinued and tachycardia maintained for another 123 to 240 seconds, after which it was discontinued and a period of recovery allowed. This sequence of observations was repeated in 3 of the 5 dogs. In another series of 9 experiments on 5 dogs (Part B, Fig. 1), the effect of comparable doses of Isuprel, administered as a 0.001% solution, on CO and MABP was first studied, then, after a period of recovery, the effect of a similar dose of the drug during ventricular tachycardia was investigated as described in the first series of experiments. This sequence of studies was also repeated in 4 of the 5 dogs. The amounts of 0.9% NaCl solution administered as vehicle for the drug have been shown previously to have no effect on the parameters studied(8).

Results. The results were strikingly constant. They are summarized in Fig. 1. In a representative experiment, illustrated by the broken lines of Fig. 1, Part B, 1.4 μg of Isuprel per kg administered over a 111 second period raised the HR from 156 to 216/min and increased CO and stroke volume (SV) from 1920 ml and 12.3 ml to 2700 ml and 12.5 ml respectively. The MABP remained essentially unchanged and TPR decreased from 83 to 60 arbitrary units. After Isuprel was discontinued, all parameters returned to or toward control values. Induction of ventricular tachycardia of a rate of 240/min resulted in a marked, sudden and immediate drop in CO and MABP, as previously reported(11). CO and MABP then returned to or toward control, and by the end of 90 seconds of tachycardia, MABP, CO, SV and TPR stabilized around values of 128 mm Hg, 1250 ml, 5.2 ml and 102 units respectively. The ventricular tachycardia being maintained, a dose of Isuprel of 1.4 μg/kg administered over a 140 second period, *i.e.,* a dose of the drug similar to the dose injected a few minutes previously, resulted in an increase in CO and a decrease in TPR similar in magnitude to the changes of CO and TPR observed during injection of a similar dose of the drug while the dog had a spontaneous sinus rhythm. However administration of Isuprel during the tachycardia definitely raised the MABP instead of lowering it or leaving it unchanged, as observed when similar doses of the drug are administered during spontaneous sinus rhythm in the present and previous experiments(5). Upon cessation of the drug administration and while ventricular tachycardia was maintained, all parameters returned to or toward the value they had during the tachycardia before injection of Isuprel. Upon cessation of ventricular tachycardia, all parameters returned to or toward their control. Similar effects were obtained when, after a period of recovery, a similar dose of Isuprel was administered again first during spontaneous sinus rhythm, then during ventricular tachycardia.

Discussion. The mechanisms of the hemodynamic effect of Isuprel alone and induced tachycardia alone have been discussed(5,11).

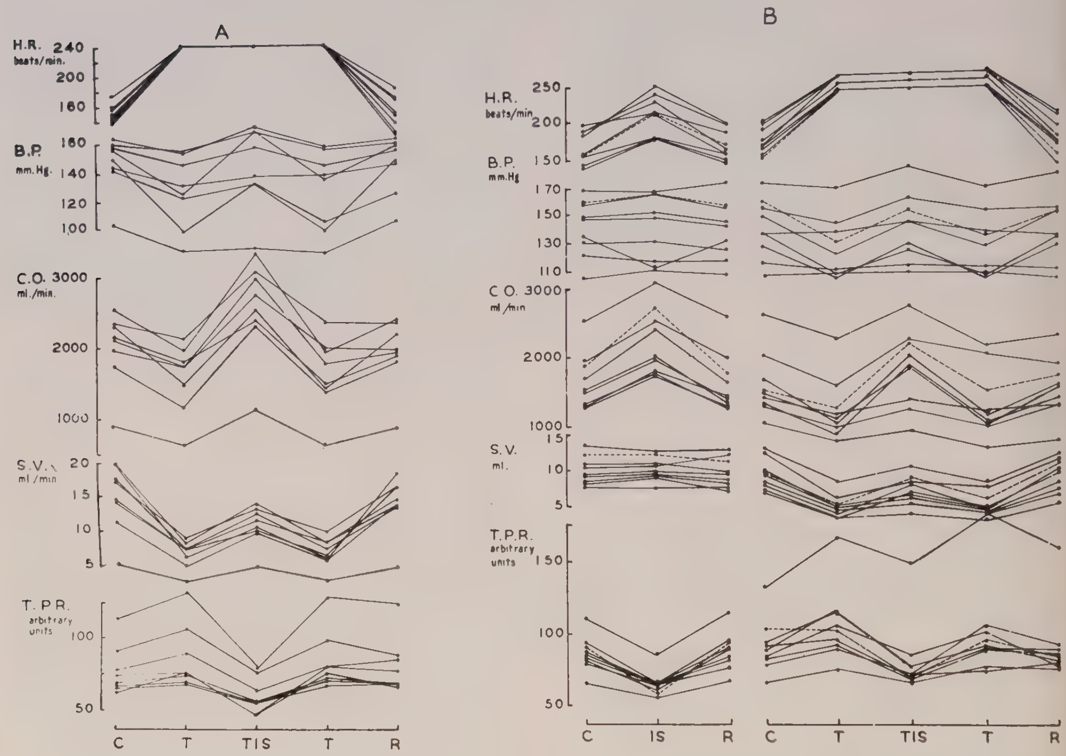

FIG. 1. Effect of isopropylarterenol hydrochloride (Isuprel®) on heart rate (HR), mean arterial blood pressure (BP), cardiac output (CO), stroke volume (SV) and total peripheral resistance (TPR). Part A pictures experiments of the first type described in text; Part B, experiments of second type, in which Isuprel was injected first during spontaneous cardiac rhythm then during tachycardia. C, control period; T, electrically induced tachycardia; TIS, electrically induced tachycardia and administration of Isuprel; R, recovery period; IS, period of Isuprel administration. Values of HR, MABP, CO, SV and TPR during period of Isuprel administration are values obtained when continuous administration of Isuprel had resulted in a progressive rise of CO to a maximal and stable level. Values of HR, MABP, CO, SV and TPR during tachycardia are values obtained when MABP and CO had become stabilized. Values of HR, MABP, CO, SV and TPR during tachycardia and Isuprel administration are values obtained when CO and MABP had become stabilized.

When Isuprel is administered during induced tachycardia, the results are essentially similar to those observed in normal anesthetized dogs with spontaneous sinus rhythm, in which HR is raised by the drug. SV and CO rise, but since HR does not change after administration of Isuprel during tachycardia, this increase in CO is due exclusively to the increase in SV. This increase in SV is presumably due to the same mechanisms as those operating while the drug is also allowed to modify HR, *i.e.*, its direct inotropic effect on the myocardium(1-5,12) and its vasodilatation effect(1-5). The intensity of the effect of the drug on CO during tachycardia seems to be similar to that of its effect when administered to the anesthetized animal with spontaneous sinus rhythm and it would seem logical to conclude that preventing the drug from increasing HR does not keep it from increasing CO. Isuprel lowers TPR of the animal during tachycardia although MABP may be observed to rise presumably because of the increase in CO. It is not clear why the increase in CO produced by Isuprel in the anesthetized dog with normal sinus rhythm is accompanied by an unchanged or even lowered MABP whereas a similar increase in CO due to the same dose of Isuprel generally raises MABP in the anesthetized dog with a fixed HR due to electrically induced tachycardia. It must be presumed that the difference is due to the peripheral constriction caused by the reduction in CO occurring during the electrically induced tachycardia. Apparently the vasodilating effect of Isuprel does not offset completely the vasoconstriction due to the reduction of CO produced by the tachycardia, and Isuprel raises MABP when administered during tachycardia. It is possible that this increase in MABP might disappear if administration of Isuprel was continued longer than in the present experiments.

Other drugs such as nitroglycerine increase both HR and CO(13,14), apparently exclusively through their vasodilating property since they are not known to have any direct effect on the myocardium. The present experiments concerning Isuprel do not necessarily permit the conclusion that nitroglycerine, like Isuprel, would increase cardiac output even if it were prevented from increasing HR. Arteriovenous fistula is also known to increase both HR and CO(15) and the increase in HR has been proven experimentally not to be a *sine qua non* for the increase in CO(15).

Summary. The effect of Isuprel on MABP, CO and TPR has been investigated in the anesthetized dog in which the effect of the drug on HR is allowed to occur, as well as in the anesthetized dog in which electrically induced atrial or ventricular tachycardia is initiated before and maintained throughout period of action of the drug to prevent the increase in heart rate normally induced by the drug. Under the circumstances of drug dosage, rate of the induced tachycardia as well as control values of HR and CO, the effect of the drug on CO and TPR is essentially the same, whether the effect of the drug on HR is permitted or prevented.

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1. Konzett, H., *Arch. f. Exp. Path. Pharm.*, 1940, v197, 27.

2. Ahlquist, R. P., *Am. J. Physiol.*, 1948, v153, 586.

3. Aviado, D. M., Jr., Wnuck, A. L., De Beer, E. J., *J. Pharm. Exp. Ther.*, 1958, v122, 406.

4. Lands, A. M., *Pharm. Rev.*, 1949, v1, 279.

5. Nakano, J., McGiff, J. C., Zekert, H., Guggenheim, F. G., Wégria, R., *Arch. Int. Pharm. et Thér.*, to be published.

6. Wégria, R., Frank, C. W., Misrahy, G. A., Sioussat, R. S., McCormack, G. H., Jr., Sommer, L. S., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 551.

7. Wégria, R., Nakano, J., McGiff, J. C., Rochester, D. F., Blumenthal, M. R., Muraviev, T., *Am. J. Physiol.*, 1958, v193, 147.

8. Wégria, R., Nakano, J., McGiff, J. C., Muraviev, T., Zekert, H., Blumenthal, M. R., *J. Pharm. Exp. Ther.*, 1959, v126, 164.

9. Shipley, R. E., Wilson, C., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 724.

10. Nakano, J., Zekert, H., Griege, C. W., Wang, K. M., Schaefer, H., Wégria, R., *Am. J. Physiol.*, 1961, v200, 413.

11. Wégria, R., Frank, C. W., Wang, H. H., Lammerant, J., *Circulation Res.*, 1958, v6, 624.

12. Remington, J. W., Hamilton, W. F., Ahlquist, R. P., *Am. J. Physiol.*, 1948, v154, 6.

13. Grollman, A., *The Cardiac Output of Man in Health and Disease*. Charles C Thomas, Springfield, Ill., 1932.

14. Wégria, R., Nickerson, J. L., Case, R. B., Holland, J. F., *Am. J. Med.*, 1951, v10, 414.

15. Frank, C. W., Wang, H. H., Lammerant, J., Miller, R., Wégria, R., *J. Clin. Invest.*, 1955, v34, 722.

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Studies with Mouse Pituitary Thyrotropic Tumors. II. Restored Responsiveness in Semi-Responsive Strain Surgically Reduced in Size.* (26571)

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A study has been made of the effect upon responsiveness to thyroxine of surgical reduction in size of an autonomous semi-responsive thyrotropic mouse pituitary tumor which had grown so large as to be no longer suppressible by this hormone. Of the 3 types of strains of thyrotropic mouse pituitary tumor isolated by Furth(1), dependent responsive, autonomous

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semi-responsive, and autonomous non-responsive, only the autonomous semi-responsive strain changes its responsiveness to thyroid hormone with increasing size after implantation. The growth of tumors of this latter strain in radiothyroidectomized mice can be arrested by administration of thyroid hormone when the tumor is 1 cm or less in size at start of treatment; but growth continues despite treatment with the hormone if the therapy is begun when the tumors are larger(2).

Development of loss of responsiveness raises several questions. The first is whether the concentration of thyroid hormone provided to the tumor cells is a factor in responsiveness, and if so, whether enough hormone could possibly be made available to cause suppression of the larger tumors without killing the animal. Second is whether a change in the tumor, or in the host because of continued presence of the tumor, or both, accounts for the change in responsiveness with tumor growth. The fact that a large, autonomous semi-responsive tumor which has become non-responsive yields, upon implantation, tumors which behave true to form for the original strain, tends to eliminate the likelihood that development of non-responsiveness by the tumor is associated with an inherent change in the tumor cells.

Methods and results. The tumor strain L24, was made available by Dr. Furth. This strain, implanted into radiothyroidectomized mice, appears as a palpable tumor about 4 months after implantation; in intact mice, no tumors appear up to a year after implantation(2). The cells then escape from suppression of growth by thyroid hormone and develop into palpable tumors at 13 months after implantation(2).

The behavior of all strains of thyrotropic mouse pituitary tumors is extremely reproducible so that valid conclusions may be drawn from groups of 4 mice each. Nonetheless, an initial experiment was made with 2 groups of 10 mice each, then the entire experiment repeated with added control groups, using 5 mice per group. Since tumor size must be estimated by palpation until autopsy, the estimated size is somewhat inaccurate. In the second experiment, tumor weight was estimated from palpation, and at autopsy determined by weighing.

The methods used in our laboratory have been described(1,2). In both present experiments the mice were implanted with L24 tumor, 3 weeks after being placed on a standard iodine diet (Ken-L-Kibble medium ration) and 2 weeks after radiothyroidectomy. Two and one-half months later, when the tumors had reached a size approximately 2 g, sodium-L-thyroxine was placed in the Ken-L-Kibble medium ration, 700 mg/100 g feed. Ten days later, the tumors were larger in all groups (Table I).

The tumors were then reduced in size surgically to produce a palpable nubbon approximately 0.5 cm in diameter. Thyroxine feeding was continued in some groups for 30 days. Control groups were fed the diet without hormone. In the first experiment, operation was delayed 1 month during which thyroxine feeding was maintained and tumor size increased.

At 30 days after surgical reduction of tumor size, no tumor had grown, as judged both from palpation and measurement at autopsy, in thyroxine-treated animals; the tumors in untreated control mice, however, had grown and were readily palpable (Table I). In the second experiment (Table I), final tumor weights were determined and again clearly show the same changes. In the second experiment, the failure of thyroxine to inhibit growth of the larger tumors is shown, as well as the inhibition of growth by thyroxine after the tumors were surgically reduced in size (Period A, Table I). Cessation of treatment with thyroxine in these latter then resulted in rapid increase in tumor size (Periods A and B, Table I). The cut-down tumors responded to thyroxine identically with implanted tumors of the same size in unoperated mice.

Discussion and Summary. Tumor implants grown in radiothyroidectomized mice to 2 g in weight or more of an autonomous semi-responsive strain of mouse pituitary thyrotropic tumor, L24, lose their responsiveness to thyroxine. When these were reduced in size surgically to a diameter of about 0.5 cm, and the hosts fed thyroxine, no tumor became palpable over the space of 30 days, although the expected increase in tumor size occurred in

TABLE I. Responsiveness to Thyroxine of Autonomous Semi-responsive L24 Tumor before and after Surgical Reduction in Size.

	Pre-operative period					Post-operative period									
						A.					B.				
	Treatment			Avg tumor size or wt* ± S.D.		Treatment			Avg tumor size or wt* ± S.D.		Treatment			Avg tumor size or wt* ± S.D.	
Exp. No.	Type	Duration (days)	No. mice	Initial	Final	Type	Duration (days)	No. mice	Initial	Final	Type	Duration (days)	No. mice	Initial	Final
				cm	cm				cm	cm					
1	T4 + KB†	40	10	2.0	3.5	KB	30	10	.75	3.0 ± .3*					
	Idem	10	10	2.0	3.0	T4 + KB	30	10	.75	.75 ± .2*					
				g	g										
2	KB	10	5	2.5	4.8 ±.2*										
	T4 + KB	10	5	2.5	5.0 ±.2*				g	g				g	g
	Idem	10	5	2.5	3.5	KB	30	5	.5	3.7 ± .2*					
	"	10	5	2.5	3.5	T4 + KB	30	3	.5	.4 ± .1*					
						Idem	30	2	.5	.5	KB	30	2	0.5	2 ±.2*
	" ‡	10	5	.5	.5	"	30	3	.5	.7 ± .2*					
						"	30	2	.5	.5	KB	30	2	0.5	3.0 ±.3*

* All measurements estimated by palpation except where measured or weighed at autopsy as indicated by asterisks.

† KB = Ken-L-Kibble medium ration; T4 = thyroxine.

‡ Unoperated controls with implanted tumors at start of experiment of same size as those surgically reduced in size in preceding groups.

similarly operated, untreated controls. The suppressed tumors promptly grew in the thyroxine treated group when thyroxine was stopped.

The results suggest that the large non-responsive tumors had not been fundamentally altered in character and that the host mouse too had not undergone any fundamental change to account for loss of responsiveness. Decreasing concentrations of thyroxine reaching the tumor cell as the tumors grew larger would thus appear to be the most likely factor accounting for lack of responsiveness of this tumor strain. Nonetheless, provision of increased amounts of thyroxine, greater than the increase in tumor size would seem to require, fail to suppress growth of the larger tumors.† Thus more is probably involved than hormonal concentration alone: perhaps limited blood supply to the tumor, perhaps loss of exogenous excess hormone from the body through overflow and excretion into the bile(3). In any event, it would appear that lethal amounts of thyroid hormone may have to be administered systemically if the minimal concentrations required to suppress growth of the larger tumors are to be reached.

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† Grindberg, R., Werner, S. C. unpublished data.

1. Furth, J., Gadsden, E. L., Burnett, W. T., Proc. Soc. Exp. Biol. and Med., 1952, v80, 4.

2. Werner, S. C., Grinberg, R., *Cancer Research*, April 1961.

3. Albert, A., Keating, F. R., Jr., *J. Clin. Endoc. Metab.*, 1949, v9, 1406.

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Lung 5-Hydroxytryptamine and Ozone Induced Pulmonary Edema in Rats.* (26572)

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Pulmonary edema and hemorrhage are well unkown results of exposure of animals to toxic doses of ozone(1-5). Experiments on dogs by Kabins *et al.*(6) suggest that 5-hydroxytryptamine (5-HT) may have a role in formation of lung edema. Since 5-HT is present in the lungs of rats in appreciable amounts(7,8), this investigation was undertaken to study the effects of ozone induced pulmonary edema on the lung 5-HT content.

Materials and methods. Male albino Wistar rats (325-375 g) were placed in wire cages 20 × 28 × 20 cm high set upon a hardware cloth shelf within an exposure chamber, constructed of galvanized sheet steel, 76 × 76 × 79 cm high. Air, passed through a Barnebey-Cheney Model 8CF air filter was admitted at the top, directed throughout the chamber by baffles, and pumped from the chamber at each of the bottom corners. Air was changed once every minute. Ozone, produced from tank oxygen in a 12,000 volt discharge ozonizer of the type employed by Quilligan *et al.* (9), was mixed with the filtered air before entering the chamber. Air samples were taken from a point just above the animals every 30 minutes during an experiment, and ozone content was determined by the neutral buffered 1% potassium iodide method of Byers and Saltzman(10), liberated iodine being measured photometrically at 352 mμ in a Beckman DU Spectrophotometer. Ozone concentration was maintained at 6 parts per million (p.p.m.) by volume, and exposure time was 4 hours in all experiments. Control animals were handled in the same manner except that addition of ozone to the chamber was omitted. Each group consisted of 11 animals. After exposure, these animals were anesthetized with pentobarbital sodium, and the lungs were removed, weighed, and assayed for 5-HT spectrophotofluorometrically in an Aminco-Bowman Spectrophotofluorometer by the method of Bogdanski *et al.*(11). The lungs of additional control and ozone-treated groups of rats, 11 animals each, were assayed for monoamine oxidase (MAO) activity by the method of Sjoerdsma *et al.*(12) using the colorimetric procedure of Udenfriend *et al.* (13). Protein contents of all lungs in this study were measured by the method of Lowry *et al.*(14). Blood samples were taken by cardiac puncture from 2 more groups of 11 rats each, exposed as before, one group to ozone and the other to air alone; these samples were obtained immediately after exposure and assayed for 5-HT by the method described by Udenfriend *et al.*(15). In the last hour of the 4-hour exposure, a few deaths occurred among those animals subjected to ozone. These animals were removed from the chamber immediately, the assay made, and the results included in the data for that experiment except when blood 5-HT was to be determined. In this case it was necessary to expose 3 additional rats to obtain blood from living animals.

Results. Rats exposed to 6 p.p.m. ozone by volume for 4 hours showed severe pulmonary edema and hemorrhage. The effects of this exposure on lung weight, lung protein, and lung 5-HT content are summarized in Table I.

Since there was an approximately 3-fold increase in lung weights and a 2-fold increase in lung protein contents of the ozone-exposed rats, consideration of the results obtained with these animals on a "per gram of tissue" or a "per gram of lung protein" basis is misleading. Therefore, results have been related to body weight in Table I. The findings show that 4 hours exposure to 6 p.p.m. ozone resulted in significant increases in lung weight, total protein in the lung, and total lung 5-HT.

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TABLE I. Mean Lung Weight, Protein, and 5-HT of Normal Male Rats Exposed for 4 Hours to 6 p.p.m. Ozone and Air Alone.

Group	g lung/100 g body wt	g lung protein/100 g body wt	μg lung 5-HT/100 g body wt
Control	.56 ± .09	.09 ± .02	.56 ± .07
Ozone	1.50 ± .30*	.19 ± .06*	1.33 ± .24*

* P <.001.

Although no definite explanation for the 5-HT increase can be given, the increased lung weight and protein content are considered to be the results of the edema and hemorrhage. Lung MAO activities of rats receiving a 4-hour exposure to 6 p.p.m. were compared to those of rats exposed to air alone. These data are summarized in Table II.

Ozone exposure resulted in a loss of MAO activity per g of lung. However, this appears to be a dilution effect which can be attributed to edema and hemorrhage since no significant difference exists between the 2 groups when MAO activity is considered in relation to quantity of lung per 100 g body weight. No significant difference was found in whole blood 5-HT levels. The group exposed to air alone had a value of 0.31 ± 0.02 μg/ml, while the ozone-exposed group had a value of 0.32 ± 0.02 μg/ml. A total of 36 rats were exposed to 6 p.p.m. ozone. In this group there occurred 5 deaths (14%) near the end of the 4-hour exposure period.

Discussion. Associated with ozone induced pulmonary edema is an increased lung 5-HT content. The source of this increase and the problem of whether it is cause or effect of pulmonary edema are open to question. Several possible explanations for this increased 5-HT can be suggested. One possibility is that the lung of the rat under certain conditions is able to trap and store 5-HT released from other tissues and that ozone exposure in some way induces such a release. If actually operative to greater or lesser degrees in the lung and possibly other tissues as well, such a trapping mechanism might be a partial explanation of the variable sensitivity of different tissues to the 5-HT releasing property of reserpine encountered by Parratt and West (7). However, the increase in 5-HT in the edematous lung may be simply a trapping of fluid containing this amine. The changes in 5-HT content and weight of the lungs are proportionally similar, 138 and 168% respectively; therefore, the 5-HT concentration of this fluid would necessarily approach that of the lung. Since this is approximately 2.5 to 3 times the concentration found in whole blood and since essentially all blood 5-HT normally is found in the platelets and none in the plasma(16), this seems unlikely. Retention of platelets in the lungs of ozone-exposed rats would be a more reasonable explanation although failure to demonstrate a change in blood 5-HT of these animals constitutes an objection to this. Another possible cause of the increased lung 5-HT following ozone exposure may be the decreased MAO activity per g of lung. This is considered to be the result of dilution by edema and hemorrhage since no such significant change is found when evaluation is in terms of activity per lung per 100 g body weight, this latter measurement indicating destruction rather than dilution of activity. If this dilution of MAO is the sole reason for the increase of lung 5-HT, then pulmonary edema must be considered the cause rather than its effect. Still another possible answer may be that ozone exposure results in an increased activity of 5-hydroxytryptophan decarboxylase, the enzyme responsible for the decarboxylation of 5-hydroxytryptophan to 5-HT. This possibility as well as those previously mentioned are now under investigation.

TABLE II. Mean MAO Activity as Measured by 5-HT Utilization in Lungs of Normal Male Rats Exposed for 4 Hours to 6 p.p.m. Ozone and Air Alone.

Group	Moles × 10^{-5} consumed/hr Per g lung	 Per lung/100 g body wt
Control	1.03 ± .21	.56 ± .14
Ozone	.33 ± .20*	.46 ± .24

* P <.001.

Summary. Rats exposed to 6 p.p.m. ozone by volume for 4 hours developed severe pulmonary edema and hemorrhage with significant increases in lung weight, lung protein content, and lung 5-HT when related to body weight. Ozone exposure was found to decrease MAO activity; this was an effect of dilution by edema rather than destruction of the enzyme. Ozone exposure did not alter blood 5-HT content. Possible mechanisms involved in the lung 5-HT increase after exposure to ozone were discussed briefly.

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1. Mittler, S., Hedrick, D., King, M., Gaynor, A., *Indust. Med. & Surg.*, 1956, v25, 301.

2. Mittler, S., King, M., Burkhardt, B., *A.M.A. Arch. Indust. Health,* 1957, v15, 191.

3. Stokinger, H. E., *A.M.A. Arch. Indust. Hyg. & Occup. Med.*, 1954, v9, 366.

4. Stokinger, H. E., Wagner, W. D., Wright, P. G., *A.M.A. Arch. Indust. Health,* 1956, v14, 158.

5. Stokinger, H. E., *ibid.*, 1957, v15, 181.

6. Kabins, S. A., Molina, C., Katz, L. N., *Am. J. Physiol.*, 1959, v197, 955.

7. Parratt, J. R., West, G. B., *J. Physiol.*, 1957, v137, 179.

8. Weissbach, H., Waalkes, T. P., Udenfriend, S., *Science*, 1957, v125, 235.

9. Quilligan, J. J., Boche, R. D., Falk, H. L., Kotin, P., *A.M.A. Arch. Indust. Health,* 1958, v18, 16.

10. Byers, D. H., Saltzman, B. E., *Am. Indust. Hyg. Assn. J.*, 1958, v19, 251.

11. Bogdanski, D. F., Pletscher, A., Brodie, B. B., Udenfriend, S., *J. Pharmacol. Exp. Therap.*, 1956, v117, 82.

12. Sjoerdsma, A., Smith, T. E., Stevenson, T. D., Udenfriend, S., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 36.

13. Udenfriend, S., Weissbach, H., Clark, C. T., *J. Biol. Chem.*, 1955, v215, 337.

14. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *ibid.*, 1951, v193, 265.

15. Udenfriend, S., Weissbach, H., Brodie, B. B., 1958, in *Methods of Biochemical Analysis,* vVI (ed. D. Glick), New York: *Interscience,* pp. 95-130.

16. Udenfriend, S., Weissbach, H., *Fed. Proc.*, 1954, v13, 412.

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Passage of Thiopental and Barbital Into Ocular and Cerebrospinal Fluids of Dog.* (26573)

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The passage of barbital and thiopental into ocular and cerebrospinal fluids was investigated in order to extend previous studies of the passage of barbiturates into various compartments such as brain, cerebrospinal fluid, fat and muscle(1-3). The comparison between ocular and spinal fluid was made because embryological and physiological similarities exist between the 2 fluids(4).

Methods and materials. Barbital was determined by the method of Giotti and Maynert(5). Thiopental was measured by the method of Brodie *et al.*(1). When concentrations were low, after extraction of drug by the organic solvent, the drug was extracted into 1 or 2 ml of aqueous phase and measured either in micro or semi-micro cuvettes. The methods gave negligible blank values and recoveries averaged 90-100%.

Mongrel, adult female dogs weighing 7-20 kg were used for this study. Barbital sodium (100 mg/kg) as a 10% neutral solution or thiopental sodium (50 mg/kg) as an alkaline (pH 10.5) 2.5% solution was injected intravenously within 5 seconds. Artificial respiration was given to the thiopental-treated dogs

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TABLE I. Passage of Barbital into Cerebrospinal and Ocular Fluids of Dogs.
Barbital sodium, 100 mg/kg i.v.

	Conc. of barbital sodium			Concentration ratios	
Time, min.	Plasma, mg/l	Cerebrospinal fluid, mg/l	Ocular fluid, mg/l	CSF/P	OF/P
9	200	31	47	.16	.24
9	220	48	64	.22	.29
30	150	95	81	.62	.54
30	130	52	64	.40	.49
60	100	97	110	.98	1.1
60	120	110	110	.92	.92
180	94	87	110	.95	1.2
180	130	120	140	.92	1.1

by rhythmic inflation of the chest with room air through an endotracheal tube fitted with a non-rebreathing valve. Adequate thoracic excursions were maintained at a rate of 16-20 per minute until satisfactory spontaneous respiration was resumed. At various time intervals the animals were sacrificed by rapid intravenous injection of 4 to 6 ml of a saturated solution of potassium chloride which produced cardiac standstill within 5 seconds. Heart blood was immediately obtained by cardiac puncture and cerebrospinal fluid was withdrawn from the cisterna magna. Ocular fluid was sampled from anterior chamber of the eye by means of 26 gauge hypodermic needle. The needle was inserted through the cornea near its lateral margin and advanced between the cornea and the iris until the tip was lying free just in front of the lens. Gentle aspiration permitted removal of 0.5 to 1.0 ml of clear ocular fluid. Each eye was used only once as a sample source. The ocular fluid value reported in Tables I and II is the average value; concentrations in the 2 eyes differed within experimental error (4-5%).

Results. Barbital concentrations in plasma were lower the longer the time interval after drug administration (Table I). Ocular fluid/plasma concentration ratios were low at first and rose to maximal values in about 60 minutes. Concentrations half that of plasma were reached in about 30 minutes. Cerebrospinal/fluid plasma concentration ratios were generally slightly lower than ocular fluid/plasma concentration ratios. Results of similar experiments performed with thiopental are shown in Table II. For thiopental, concentration ratios were corrected for the extensive binding to plasma proteins. After making this correction, concentration ratios for thiopental were found much higher than for barbital at 9 minutes. Cerebrospinal fluid/plasma water concentration ratios were consistently (in contrast to barbital) higher at 3 and 9 minutes than ocular fluid/plasma water concentration ratios. The data in Tables I and II indicate that thiopental enters ocular and cerebrospinal fluid more rapidly than barbital and that cerebrospinal fluid/plasma and ocular fluid/plasma ratios of about one eventually are achieved for both drugs.

Discussion. Some of the pertinent physical properties of the 2 barbiturates chosen for this study had been determined previously. The pKa is 7.8 for barbital and 7.6 for thiopental. The partition coefficient between peanut oil and pH 7.4 phosphate buffer was

TABLE II. Passage of Thiopental into Cerebrospinal and Ocular Fluids of Dogs.
Thiopental sodium, 50 mg/kg i.v.

	Concentrations of thiopental sodium			Concentration ratios	
Time, min.	Plasma,* mg/l	Cerebrospinal fluid, mg/l	Ocular fluid, mg/l	CSF/Plasma water	OF/Plasma water
3	72 (18)	13	6.3	.72	.35
3	94 (24)	8.2	6.1	.35	.25
9	67 (17)	11	6.5	.65	.38
9	62 (16)	14	8.7	.88	.54
30	33 (8.3)	11	9.2	1.3	1.1
30	—	20	12	—	—
30	50 (13)	13	11	1.0	.85
30	23 (5.8)	8.3	6.3	1.4	1.2

* Values in parentheses are calculated values of drug concentrations in plasma water based on 75% binding to plasma protein.

0.23 for barbital as compared with 63 for thiopental(2). Based upon the Henderson-Hasselbalch equation, at pH 7.4, 41% of barbital is present in the unionized form while at the same pH 61% of the thiopental present is unionized. Barbital is not appreciably bound to plasma protein whereas thiopental is extensively bound (65-75%)(9-11). It is evident that the 2 barbiturates differ most strikingly in lipid solubility and in the extent of their binding onto plasma protein; at pH 7.4 both are unionized to about the same extent.

Based upon studies in the rabbit, Mayer, Maickel and Brodie(12) calculated relative permeability constants for penetration into cerebrospinal fluid by various drugs. For thiopental and barbital, they found average constants of 0.69 and 0.029 min^{-1} respectively. These authors suggested that the blood-brain and blood-spinal fluid barriers behave as a lipid boundary which drugs cross by simple diffusion, according to the extent of their dissociation and the lipid solubility of the unionized molecules. These authors (13) have also shown that thiopental leaves the cerebrospinal fluid more rapidly than barbital. Recently, Brodie and co-workers extended their investigations, using the dog (14); permeability constants (cerebrospinal fluid) were similar to those found for rabbits —0.026 for barbital and 0.50 min^{-1} for thiopental, respectively. The present results with cerebrospinal and ocular fluids are consistent with these findings, in that barbital was found to enter both fluids more slowly than thiopental.

Just as there is a blood-brain barrier and a blood-spinal fluid barrier, there is also a blood-ocular fluid barrier to the free passage of certain substances from the general circulation into the intraocular fluid. Ross(6) has suggested that lipid solubility is of greater importance than molecular size in the passage of non-electrolytes into ocular fluid. Other authors(7,8) are in agreement with this concept though factors such as pKa(14,15), diffusion coefficient and state of hydration may also be involved. According to Davson (7), there are two processes for penetration of substances from the blood into ocular fluid: (a) the secretory flow mechanism and (b) the iris diffusion mechanism. The secretory mechanism is associated with the ciliary process and the relatively small posterior chamber; the diffusion mechanism is associated with the iris and the relatively large anterior chamber. These mechanisms involve passage of substances through one or more membranes. Davson(7) has suggested that, for a series of compounds of comparable molecular size, rate of transfer by both mechanisms increases with increase in lipid solubility. However, rate of transfer by the secretory flow mechanism soon reaches a maximum which is probably determined by rate of formation of ocular fluid itself by this process. Beyond this point, a further increase in total transfer rate can still result from further increase in lipid solubility, due to increased transfer by the iris diffusion mechanism. Under the experimental conditions used, thiopental enters both ocular and cerebrospinal fluids more rapidly than barbital. From these data and the works cited above it seems reasonable to implicate high lipid solubility as the key factor in rapid distribution of thiopental into these body compartments. Simple diffusion across lipoid boundaries may be the major mechanism involved.

Summary. Experiments with dogs have shown that thiopental enters ocular fluid and spinal fluid more rapidly than barbital does. This is probably due to the higher lipid solubility of the thio barbiturate.

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1. Brodie, B. B., Mark, L. C., Lief, P. A., Bernstein, E., Rovenstine, E. A., *J. Pharm. & Exp. Therap.*, 1950, v98, 85.

2. Mark, L. C., Burns, J. J., Brand, L., Campomanes, C. I., Trousof, N., Papper, E. M., Brodie, B. B., *ibid.*, 1958, v123, 70.

3. Mark, L. C., Brand, L., Dayton, P. G., Taller, D., Papper, E. M., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 606.

4. Wegefarth, P., Weed, H. L., *J. Med. Res.*, 1914, v31, 167.

5. Giotti, A., Maynert, E. W., *J. Pharm. & Exp. Therap.*, 1953, v101, 26.

6. Ross, E. J., *J. Physiol.*, 1951, v112, 229.

7. Davson, H., Physiology of the Ocular and Cerebrospinal Fluids, Little, Brown and Co., Boston, 1959.

8. Langham, M. E., *Physiol. Rev.*, 1958, v38, 215.

9. Goldbaum, L. R., Smith, P. K., *J. Pharm. & Exp. Therap.*, 1954, v111, 197.

10. Taylor, J. D., Richards, R. K., Davin, J. C., Asher, J., *ibid.*, 1954, v112, 40.

11. Schanker, L. S., Shore, P. A., Brodie, B. B., Hogben, A. M., *ibid.*, 1957, v120, 528.

12. Mayer, S. E., Maickel, R. P., Brodie, B. B., *ibid.*, 1959, v127, 205.

13.———, *ibid.*, 1960, v128, 41.

14. Brodie, B. B., Kurz, H., Schanker, L. S., *ibid.*, 1960, v130, 20.

15. Zubrod, C. G., Rall, D. P., *ibid.*, 1959, v125, 194.

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Studies of Alcaptonuria: Collagenase Degradation of Homogentisic-Tanned Hide Powder Collagen.* (26574)

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Studies in this laboratory on the pathogenesis of alcaptonuric arthritis, the third of the 3 temporally related, characteristic components of the alcaptonuria syndrome (homogentisic aciduria, or alcaptonuria; ochronosis; alcaptonuric, or ochronotic arthritis) have been concerned primarily with the interactions of various homogentisic acid solutions with collagen. Since it had been shown previously(1) that homogentisic acid does not interact with unbound chondroitin sulfate, it followed logically that interactions with collagen, the other and major component of cartilage, as well as the other connective tissues, necessarily was implicated.

Indeed, it was later shown(2,3) that homogentisic acid autoxidation polymers (HGA-OP), in diametric opposition to unoxidized, monomeric homogentisic acid solutions (HGA), become more or less irreversibly bound to collagen by a pH-independent reaction. For steric reasons, this results in significant cross-linking of adjacent collagen polypeptide chains and stabilization of the collagen structural lattice. As such, the resulting tanning reaction would be expected to result in a product which would not only resist water solubilization and heat degradation, as has been previously demonstrated, but would also be expected to result in increased resistance to the action of a number of proteolytic enzyme systems. The present report notes the result of one such study.

Material and methods. Twelve gram batches of limed and neutralized (pH 5.4) bovine hide powder collagen (American Standard Hide Powder, purchased from Frank F. Marshall, Ridgeway, Pa.) were passed through No. 20 ASTM sieves, placed in covered Ehrlenmeyer flasks and reacted for approximately 75 hours at room temperature (21°-28°C) with 500 ml aliquots of water or of aqueous, autoxidized, polymeric homogentisic acid (HGA-OP) solutions (3 mg/ml), prepared as outlined previously(4). The liquid phase was removed by suction filtration after the period of interaction and the solid (collagen) phase was freeze-dried. (A considerable quantity of the previously untreated hide powder collagen was lost by solubilization during water treatment, whereas significantly smaller amounts were solubilized during interaction with HGA-OP. No attempt was made here, however, to provide quantitative data on the precise degree of solubilization in either instance.)

Two hundred milligram samples of each collagen preparation were placed into dry, chemically clean screw-top 15 ml graduated

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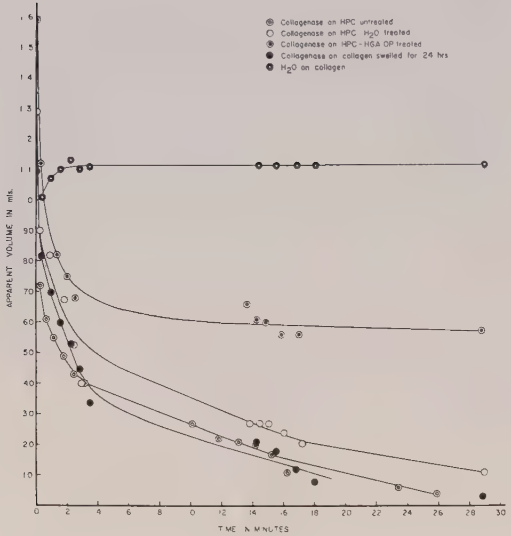

FIG. 1.

centrifuge tubes. The collagen preparations were: (1) untreated, isoelectric hide powder collagen, directly suspended in 10 ml water (pH 6.10); (2) untreated, isoelectric hide powder collagen permitted to swell in 10 ml H_2O for 24 hours before exposure to collagenase; (3) untreated, isoelectric hide powder collagen, directly suspended in the collagenase solutions; (4) isoelectric hide powder collagen pre-treated with water (pH 6.10) as outlined here, then swelled in water for 24 hours before interaction with collagenase, and (5) HGA-OP pre-treated hide powder collagen, prepared as outlined here, then permitted to swell in water at pH 6.10 for 24 hours before exposure to the collagenase solution.

After treatment as outlined immediately above, 10 ml of a collagenase solution (4 mg/ml, lot No. 5732, purchased from Worthington Biochemical Corp., Freehold, N. J.) were then layered on to certain of the collagen preparations and thoroughly stirred to effect dispersion of the solid phase. At appropriate time intervals, the tubes were centrifuged at 2500 rpm at room temperature for 5 minutes. The mean apparent hydrated volume of the solid phase (minimum of 5 tubes) was read to the nearest 0.1 ml and estimated to the nearest 0.05 ml from the graduations on the side of each tube. The tubes were then restirred and permitted to stand in air until the next determination was made.

Initial pH (pH_i) and final pH (pH_f) values of the enzyme solutions were determined with a Beckman Zeromatic pH meter (instrumental error, 0.03 pH).

Results. The mean apparent hydrated volume of the solid (collagen) phase in presence of water and collagenase solutions is plotted against time of interaction in Fig. 1 and summarized in Table I.

Discussion. A number of investigators have suggested certain technical problems, reviewed in detail by Robb-Smith(5) and Gus-

TABLE I. Summary of Observations.

Collagen phase	Liquid phase	Treatment	pH_i	pH_f	ΔpH	Mean app. vol (ml at 1400-1800 min.)	% Δ
Untreated H.P.C.	H_2O	Direct. susp.	6.10	5.00	−1.10	1.11	—
Idem	Collagenase	*Idem*	6.07	6.22	+ .15	.14	87.4
”	”	H_2O-swelled	6.05	6.65	+ .60	.15	86.5
H_2O-treated H.P.C.	”	*Idem*	5.90	6.25	+ .35	.24	78.4
HGA-OP-treated H.P.C.	”	”	5.90	5.48	− .42	.60	45.9

“H.P.C.” signified limed and neutralized (isoelectric) bovine hide powder collagen. “Direct. susp.” indicates that hide powder collagen preparation was directly suspended in liquid phase. “H_2O-swelled” indicates that collagen preparation was permitted to swell in water (pH 6.01) for 24 hr before water was decanted and collagenase solution added. pH_i is initial and pH_f final pH of liquid phase. “Mean app. vol” = mean apparent hydrated vol, used here as a convenient and rough estimate of relative effects of water and collagenase on various hide powder collagen preparations, and arbitrarily taken (mean value of all apparent volume determinations) between 1400 and 1800 min. after beginning of interaction with collagenase. “Percent change” is presented in terms of calculated percentage change in mean apparent hydrated volume of collagenase-treated preparations relative to that of untreated hide powder preparation directly suspended in water.

tavson(6,7), to indicate that mammalian hide powder collagen, in contradistinction to native collagen, is not a suitable agent for investigation of proteinase action. This is largely attributable to the facts that hide powder contains a mixture of both native and heat denatured collagen, that "limed skin tissue(pelt) used in the isoelectric range is too resistant," and, in general, that any pretreatment of native collagen necessarily imparts a modification of molecular structure and organization, and hence of the properties of native collagen.

For the present basically qualitative purposes, however, a precise knowledge of the chemical nature of either starting material or split-products would not be necessarily critical. If a tanning phenomenon does occur when limed and neutralized hide powder collagen is interacted with autoxidized, polymeric homogentisic acid (HGA-OP) solutions, then by definition, it is required only to demonstrate that the pre-treated product (HGA-OP bound collagen) possesses a significantly increased resistance to collagenase in comparison to that of the untreated product. This is precisely what was observed.

Analysis of the kinetic data clearly indicates that pH *per se* is not a factor in the observed changes since there was a distinct difference in the effects of collagenase solutions and water controls at exactly the same initial pH. Furthermore, since only a negligible and probably insignificant final effect could be attributed to swelling of the isoelectric collagens in water before addition of collagenase, it follows that this experimental procedure is likewise not determining in the present observations. It appears, therefore, that the measured differences in rate, magnitude and calculable percentage change in apparent hydrated volume of the untreated and HGA-OP treated collagens in the presence of collagenase are highly significant.

Thus, in expansion of earlier data and in support of additional (unpublished) data indicating decreased chemical reactivity of collagen in consequence of treatment with HGA-OP, it appears that HGA-OP acts in all classical manners as a potent tanning agent for hide powder collagen.

Summary. A volumetric study has been conducted of the effects of collagenase solutions on isoelectric and treated bovine hide powder collagen preparations. The data indicate that the enzyme solution used is active against hide powder collagen, that the effects of the collagenase noted here are independent of pH and that pretreatment of hide powder with the autoxidation polymers of homogentisic acid (HGA-OP) results in significant diminution of the effects of collagenase. With earlier data indicating that HGA-OP binds to hide powder, the current data are interpreted as indicating that HGA-OP acts as an effective tanning agent for hide powder collagen.

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1. Milch, R. A., Titus, E. D., *Arthritis & Rheumat.*, 1958, v1, 566.
2. Milch, R. A., *Surg. Forum,* 1960, v11, 448.
3. Milch, R. A., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 68.
4. Milch, R. A., Titus, E. D., Loo, T. L., *Science,* 1957, v126, 209.
5. Robb-Smith, A. H. T., in *Nature and Structure of Collagen,* J. T. Randall, Ed., Academic Press, New York, 1953, 14-26.
6. Gustavson, K. H., *The Chemistry and Reactivity of Collagen,* Academic Press, New York, 1956.
7. ———, *The Chemistry of Tanning Processes,* Academic Press, New York, 1956.

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Action of the Kallikreins on Synthetic Ester Substrates. (26575)

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The kallikreins are hypotensive proteinases of animal origin which cause a marked fall in blood pressure and stimulate certain smooth muscles(1). They exert their hypotensive effect by enzymatic action on kallidinogen, an α-2 globulin found in plasma, to release the polypeptide kallidin. Although it is likely that the kallikreins derived from various sources such as the urine, saliva, plasma and pancreas, may attack the same substrate in plasma to release the same polypeptide, they are not identical proteolytic enzymes since they vary in susceptibility to the action of various proteolytic inhibitors(1,2,3). Recently, Contzen, *et al.*(4), Habermann(5) and Werle and Kaufmann-Boetsch(6) have presented evidence that one or more of the kallikreins are capable of attacking various arginine esters. This report describes the action of 4 of the kallikreins derived from hog pancreas, human pancreas, human urine and human plasma on a number of synthetic ester substrates.

Materials and methods. Highly purified *hog pancreatic kallikrein* (240 Frey units/mg) was kindly supplied by Dr. H. Moriya (7). Crude *human pancreatic kallikrein* (4 Frey units/mg) was prepared by adsorption on diethylaminoethyl cellulose (DEAE-C) as previously described(3). *Human urinary kallikrein* from 150 liters of human urine was adsorbed on Amberlite XE-64 (IRC-50) at pH 4.0(3) and eluted with 10 liters of 0.1 M sodium phosphate buffer, pH 6.0. The eluate was diluted to 32 liters with water and treated with 22.5 g of 0.36 meg/g DEAE-C prepared from Whatman Ashless Cellulose Powder, Standard Grade, by the method of Ellis and Simpson(8). The pH was adjusted to 6.7 with hydrochloric acid and the mixture was stirred for 2 hours at 15-20° and allowed to settle overnight. The adsorbent was removed by filtration, washed on the filter with distilled water, and eluted with about 500 ml of 0.2 M sodium phosphate buffer, pH 7.0, containing 0.5 M sodium chloride. The dialyzed and freeze-dried eluate represented 80-100% yield of the kallikrein at 6 Frey units/mg. *Human plasma kallikrein* was activated by addition of acetone as previously described(3). The solution was dialyzed to remove salts and acetone, and the precipitate which appeared on dialysis was removed by filtration. The clear supernatant was diluted with distilled water to 5 volumes (based on the original plasma volume of 1.4 liters); 73.5 g 0.84 meg/g DEAE-C were added; the pH adjusted to 8.0 and the mixture was stirred for 1 hour at 12°. The inactive adsorbate was removed by filtration and the clear supernatant stirred with an additional 71 g DEAE-C except that pH was adjusted to 6.5. The second inactive adsorbate was removed by filtration and the plasma kallikrein was adsorbed from the supernatant by addition of 70.5 g of 0.36 meg/g carboxymethyl cellulose (CM-C). The pH was adjusted to 6.0 with sodium hydroxide and the mixture was stirred for 1 hour at 12°. The adsorbent was recovered by filtration, washed with about 1 liter of distilled water and eluted with 500 ml of 0.3 M potassium phosphate buffer, pH 7.0. The eluate was adjusted to pH 6.9, dialyzed against running tap water for 16 hours at 15-17° and dried from the frozen state. A 15-25% yield of kallikrein with a potency of 0.3 Frey units/mg was obtained by these procedures. *Esterase* activity of the kallikrein preparations was studied using Robert's modification of the Hestrin method(9). To tubes containing 1.0 ml of .06 M ester and 1.0 ml of 0.75 M tris (hydroxymethyl) amino methane (Tris) buffer was added 1.0 ml of enzyme solution. After incubation at 37° the amount of residual ester was determined colorimetrically as previously described(9), except that the precipitate following addition of the trichloroacetic acid was removed by filtration. The highly purified hog pancreatic kallikrein and the crude human urinary kallikrein did not precipitate at this

TABLE I. Ability of Kallikreins to Digest Synthetic Esters.†

Substrate .02 M, pH 7.5	Kallikreins derived from Urine 6 FU‡	 Pancreas 6 FU	 Plasma 1.7 FU
Benzoyl-L-arginine methyl ester	5.4	14.9	11.2
Benzoyl-L-arginine ethyl ester	5.3	16.2	10.6
p-Toluenesulfonyl-L-arginine methyl ester	8.8	7.5	10.5
Benzoyl-dl-alanine methyl ester*	<1	<1	<1
Benzoyl-dl-alanine ethyl ester*	<1	<1	<1
L-leucine ethyl ester	<1	<1	<1
L-lysine methyl "	<1	<1	<1
L-tyrosine " "	<1	1.5	<1
Acetyl-L-tyrosine ethyl ester*	<1	11.2	<1
Acetyl-L-tryptophan ethyl ester*	<1	1.2	<1

* Esters dissolved in 25, 30, 15 and 50% methyl cellosolve, respectively. Determinations of these proteinases with TAMe in presence of 50% methyl cellosolve caused some inhibition of the enzymes as only 2.1, 3.2 and 9.4 μmoles were digested, respectively.

† μmole ester digested per 60 min., 37°.

‡ FU (Frey Units) of biological activity.

concentration of trichloroacetic acid. Inhibitors were dissolved in the Tris buffer and incubated with enzyme at 37°. The enzymatic reaction was initiated by addition of substrate. The *kallikrein* content of the various preparations was determined by measuring the increase in blood flow following injection of .01-.04 Frey units in the dog(3). Because the response to plasma kallikrein as compared to pancreatic kallikrein varied from one dog to another, a separate plasma kallikrein standard based on the average response furnished by 3 dogs was utilized for this proteinase. Crystalline soy bean trypsin *inhibitor* (SBTI) was purchased from Worthington Biochemical Corp. Pancreatic trypsin inhibitor (PTI) was a product of Nutritional Biochemicals Corp. Diisopropylfluorophosphate was purchased from K & K Laboratories, Inc.

Results. The esterase action of purified hog pancreatic kallikrein, crude human urinary kallikrein and human plasma kallikrein is shown in Table I. All 3 proteolytic solutions were capable of digesting arginine esters. Highly purified hog pancreatic kallikrein also gave some digestion of tyrosine and tryptophan esters, and acetyl-L-tyrosine ethyl esters appeared to be as good a substrate for this proteinase as the arginine esters. Urinary and plasma kallikrein, on the other hand, did not attack the aromatic substrates. Other esters, *i.e.*, alanine, leucine, lysine, were not attacked by the kallikrein solutions.

It is well established that certain inhibitors compete with the substrate for the active site of the enzyme. If the reversible plasma inhibitor of urinary and pancreatic kallikreins (10) is a competitive inhibitor, the presence of those esters which are substrates for the kallikreins should prevent the inhibition of the biological activity by the inhibitor. These studies should provide, despite the crudeness of the kallikrein preparations, more direct evidence that the kallikreins were actually capable of digesting arginine esters. In the absence of esters greater than 50% inhibition of crude human urinary and pancreatic kallikreins by the plasma inhibitor was obtained (Table II). However, in presence of the arginine esters the inhibition was competitively blocked. Leucine and lysine esters, on the other hand, failed to prevent the inhibition of the kallikreins. Benzoyl-L-arginine amide also competes with the inhibitor and may be a substrate for the kallikreins. Similar measurements were also made with human plasma kallikrein using diisopropylfluorophosphate as the inhibitor. Highly purified hog pancreatic kallikrein gave results in no way different from those reported for crude human pancreatic kallikrein. However, the crude human pancreatic kallikrein, at equivalent biological activity, was much more potent in its action on synthetic esters *in vitro* and is probably contaminated by other pancreatic proteinases. These data are compatible with the kallikreins as proteolytic enzymes capable of digesting arginine esters. The direct competitive inhibition of the kallikreins by these synthetic amino acid esters cannot be satisfactorily tested *in vivo*. Attempts to measure the plasma inhibitor to human urinary kallikrein or hog pancreatic kallikrein *in vitro* at pH 7.5 have shown that addition of 6 Frey units to a

TABLE II. Ability of Esters to Inhibit Plasma Inhibition of Human Urinary and Pancreatic Kallikrein and DFP Inhibition of Plasma Kallikrein.

Esters, .02 M	% inhibition by plasma of kallikreins derived from Urine	Pancreas	% inhibition by DFP of plasma kallikrein
None	>50	>50	>50
Benzoyl-L-arginine methyl ester	12	2	50
" ethyl "	20	0	8
p-Toluenesulfonyl-L-arginine methyl ester	8	29	20
L-leucine ethyl ester	>50	>50	>50
L-lysine methyl "	>50	>50	>50

To out-dated human ACD (acid citrate dextrose) plasma was added 3 volumes of 0.75 M Tris buffer, pH 7.5, and 2 volumes of enzyme at 1.5 Frey units/ml. Solutions were incubated for 2 hr at 37° in presence or absence of esters. To 1.0 ml of human plasma kallikrein, 0.6 Frey units in 0.1 M Tris buffer, pH 7.5, was added 1/10 volume of 1×10^{-3} M DFP (diisopropylfluorophosphate) in anhydrous isopropanol and solutions incubated for 1½ hr at 37° in presence or absence of esters. In presence of benzoyl-L-arginine amide 49, 22 and 60% inhibition of the 3 kallikreins was obtained, respectively.

1:2 dilution of plasma in Tris buffer caused little or no inhibition of the p-toluene sulfonyl-L-arginine methyl ester (TAMe) activity after incubation for 2 hours at 37°. These results are in agreement with the biological data, and suggest that TAMe effectively competes with the reversible plasma inhibitor.

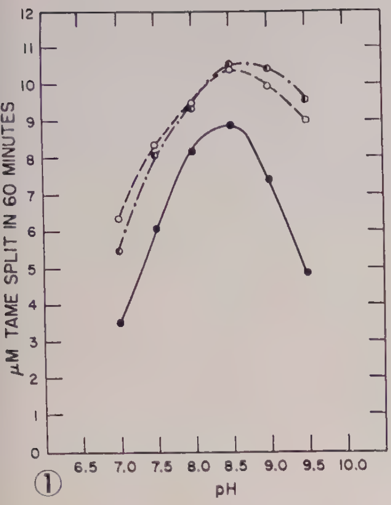
Because TAMe appeared to give the same esterolytic activity at equivalent biological activity with both urinary and pancreatic kallikreins (Table I), this substrate was chosen for more detailed investigation. When the μmoles of TAMe digested in 60 minutes are plotted against pH (Fig. 1), the pH optima of the 3 proteinases in Tris buffer was found to be 8.5. TAMe itself undergoes a small digestion at the higher pH's and the figures reported have been corrected for the decrease in the blank. Comparison of the μmoles of TAMe digested in 60 minutes with the biological activity in terms of Frey units (Fig. 2) showed that the hog pancreatic and human urinary kallikreins digested identical quantities of TAMe at pH 8.5 except in the higher regions around 9 Frey units/ml. Plasma kallikrein, on the other hand, appeared to be a much more potent protease against this synthetic ester, requiring about ⅕ of the Frey units to liberate the same μmoles of TAMe.

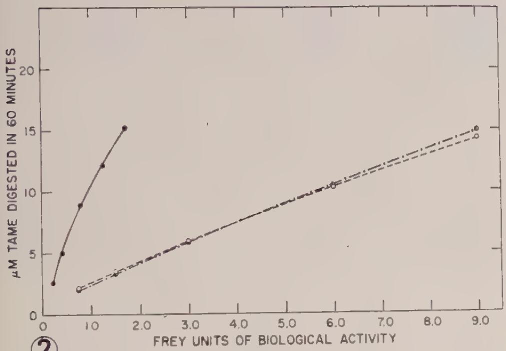
In an attempt to ascertain how much of

TABLE III. Comparison of % Inhibition of Esterase and Biological Activities of the Kallikreins by Trypsin Inhibitors.

	% inhibition at pH 8.5 of kallikreins derived from					
	Human urine		Hog pancreas		Human plasma	
Inhibitors, μg/ml	Esterase	Biological	Esterase	Biological	Esterase	Biological
SBTI .25					24	
.5					47	
1					73	18
2						45
4						>50
500	0	0	0	0		
PTI .5	23	20		20		
1	46	44	17	35		
2	73	>50	31	>50		32
4			50			56
8						>50
10					29	
20					45	
40					60	

Inhibitors were incubated with the kallikreins for 15 min. at 37°. Urinary and pancreatic kallikreins were inhibited at 3 Frey units/ml and plasma kallikrein at 0.6 Frey unit/ml. Soy bean trypsin inhibitor (SBTI). Pancreatic trypsin inhibitor (PTI).





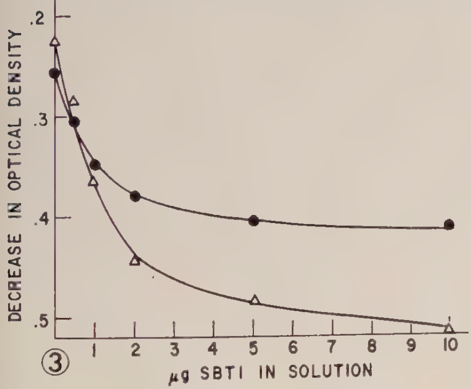


FIG. 1. Effect of pH on digestion of p-toluene sulfonyl-L-arginine methyl ester (TAMe) by the kallikreins; ◒—•—◒, hog pancreatic kallikrein; ○——○, human urinary kallikrein; ●——●, human plasma kallikrein.

FIG. 2. Comparison of biological and esterase activities of the kallikreins; ◒—•—◒, hog pancreatic kallikrein; ○——○, human urinary kallikrein; ●——●, human plasma kallikrein.

FIG. 3. Ability of soy bean trypsin inhibitor (SBTI) to inhibit 0.3 ml of acetone-activated and dialyzed plasma, ●——●, and partially purified plasma kallikrein, △——△, 4.2 mg. Addition of 20 and 100 μg SBTI gave little increase in inhibition.

TAMe activity was due to kallikreins and how much could be due to possible contamination with other proteases, the same concentration of kallikrein was compared by the esterase and biological methods for its ability to be inhibited by SBTI and PTI trypsin inhibitors. Crude human urinary kallikrein gave identical percentage of inhibition with both procedures (Table III). However, with the highly purified hog pancreatic kallikrein, an approximately 2-fold discrepancy was found with PTI inhibitor. This would suggest that even at this great purity other proteases from the pancreas were still present as contaminants. Neither biological nor esterase activity was inhibited with SBTI, which would indicate that trypsin was not one of the contaminating enzymes. Crude human plasma kallikrein was most grossly contaminated by other esterolytic activity as determined by this procedure. The esterolytic activity required about 4 times as much SBTI as did the biological activity and was much more sensitive to PTI. The ratio of inhibition of plasma kallikrein by SBTI and PTI was found in these studies to be 1:3 as compared to the 1:10 found with an earlier preparation(3). It is possible, therefore, that each lot of crystalline PTI will vary in its ability to inhibit the kallikreins. Investigation of the esterases liberated by acetone activation of plasma showed that, unlike the partially purified plasma kallikrein, not all TAMe activity could be inhibited by SBTI (Fig. 3). These data were a clear indication that at least 2 esterases were activated by acetone. During the purification of the acetone-activated and dialyzed plasma, it was found that a portion of the esterase activity, which was not inhibited by SBTI, could be adsorbed at pH 8.0 on DEAE-C and that the remainder of this activity could be removed by adsorption at pH 6.5 (Table IV). The esterase activity which was inhibitable with SBTI, on the other hand, failed to adsorb on DEAE-C at either pH and could be adsorbed from solution by CM-C at pH 6.0. The kallikrein activity paralleled the SBTI-inhibited esterase activity. Although it appears, from the com-

TABLE IV. Esterolytic and Kallikrein Activity during Purification of Human Plasma Kallikrein.

Enzyme source	Esterase activity in EU* Total	Esterase activity in EU* Inhibited by SBTI	Kallikrein activity in FU*	Protein, mg/ml†
Acetone-activated and dialyzed plasma	4.1	2.7	5.3	53
DEAE-C adsorption, pH 8.0				
Filtrate	2.3	1.5	1.2	13.8
Eluate	.98	.18	.3	27.0
DEAE-C adsorption, pH 6.5				
Eluate	.50	.11	.2	5.3
CM-C adsorption, pH 6.0				
Filtrate	.06	.06	.1	5.8
Eluate	.9	.84	.84	2.8

* Arbitrary esterase units (EU) based on Frey units (FU) found in the purest plasma kallikrein preparation (eluate from CM-C adsorption at pH 6.0).

† mg non-dialyzable protein recovered.

All results calculated/ml original concentration of plasma. Esterase activity was inhibited by addition of 100 μg soy bean trypsin inhibitor (SBTI). Acetone-activated and dialyzed plasma was fractionated by adsorption on diethylaminoethyl cellulose (DEAE-C) at pH 8.0, filtrate was then adsorbed with DEAE-C at pH 6.5, and filtrate from this adsorption was further fractionated by adsorption on carboxymethyl cellulose (CM-C) at pH 6.0.

parison of the esterase and biological activities (Table III) of this crude preparation of kallikrein, that plasma kallikrein is not responsible for all of the esterase activity, attempts to separate the kallikrein and residual esterase activity by hydroxyapatite chromatography have not been successful. To date, a single proteolytic and biologically active peak has been obtained.

It was thought possible that some of the esterases activated by acetone might be capable of digesting other synthetic ester substrates. However, when the acetone-activated and dialyzed plasma and the esterase activity prepared by adsorption on DEAE-C at pH 6.5 were tested on the various synthetic esters at a level capable of digesting 10-12 μmoles of the arginine esters, they, like the product prepared by CM-C adsorption, failed to digest the other esters.

Discussion. These data would suggest that the kallikreins are capable of digesting arginine ester. Perhaps the strongest evidence that these synthetic amino acid esters are substrates for the kallikreins has been derived from inhibition studies. It was readily demonstrated that the arginine esters acted to prement inactivation of the biological activity by their inhibitors. However, comparison of the inhibition of esterase and biological activities with the trypsin inhbtors revealed that neither the pancreatic nor the plasma kallikreins gave parallel inhibition. The data for urinary kallikrein are most promising and suggest that urinary kallikrein may be the main TAMe esterase in this preparation.

Highly purified hog pancreatic kallikrein, which has been shown to be 40% pure(7), hydrolyzes acetyl-L-tyrosine ethyl ester as well as arginine esters. Unfortunately the ability of this ester to prevent inhibition of the biological activity by the plasma inhibitor could not be tested because of its limited solubility. It is also evident from the inhibition studies with PTI that the apparent correlation of esterolytic and bioolgical activities of human urinary kallikrein and hog pancreatic kallikrein is fortuitous. Homogeneous hog pancreatic kallikrein will probably not be as potent a TAMe esterase as human urinary kallikrein.

The fact that all of the kallikreins appear to be capable of attacking arginine esters is in good agreement with the studies of Elliot, Lewis and Horton(11) who have shown that bradykinin, prepared by the action of trypsin on bovine globulin, is a linear nonapeptide containing both N-terminal and C-terminal arginine residues. If it is assumed that kallidin and bradykinin are similar or identical, the kallikreins are probably splitting kallidinogen at a specific peptide link of which arginine is one component.

The esterolytic activity of acetone-activated plasma kallikrein is of particular interest because of the many esterases which have already been tentatively identified in human plasma. Of those esterases known to be inhibited by SBTI, *e.g.*, plasmin, thrombin-E, thrombokinase and the permeability factor, only plasmin has been shown not to be activated by acetone(3). The observation that intravenous infusion of thrombin-E(12) did not alter the blood pressure in dogs clearly differentiates this proteinase from plasma kallikrein. In addition, although definitive data are lacking, it would appear unlikely that thrombokinase(14) and plasma kallikrein were identical. The premeability factor(13), on the other hand, cannot be definitely distinguished from plasma kallikrein. There are, therefore, at least 5 proteinases present in human plasma-plasmin, thrombin-E, thrombokinase, C'_1 component of complement and plasma kallikrein. To these can be added the acetone-activated proteinase(s) which are not inhibitable with SBTI since they differ from C'_1 in not attacking tyrosine esters. Austen (15) has also noted the presence in chloroform-activated human serum of a TAMe esterase which is not inhibited by SBTI.

Summary. Evidence is presented which suggests that the kallikreins derived from hog pancreas, human pancreas, human urine and human plasma are capable of digesting those synthetic esters which contain arginine as the specific amino acid residue. Highly purified hog pancreatic kallikrein also digests acetyl-L-tyrosine ethyl ester. Arginine esters and benzoyl-L-arginine amide prevented both inhibition of human urinary and pancreatic kallikreins by human plasma and inhibition of human plasma kallikrein by diisopropylfluorophosphate. Acetone activates not only plasma kallikrein but another proteolytic enzyme(s) which attack arginine esters and are not inhibited by SBTI.

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1. Werle, E., *Polypeptides Which Stimulate Plain Muscle,* 1955, E. & S. Livingstone Ltd., Edinburgh and London.

2. Werle, E., Forrell, M. M., Maier, L., *Naturwissen.,* 1954, v41, 89.

3. Webster, M. E., Pierce, J. V., *J. Pharm. Exp. Therap.,* 1960, v130, 484.

4. Contzen, Ch., Holtz, P., Raudonat, H. W., *Naturwissen.,* 1959, v46, 402.

5. Habermann, E., *Arch. Exp. Path. Pharmakol.,* 1959, v236, 492.

6. Werle, E., Kaufmann-Boetsch, B., *Z. Physiol. Chem.,* 1960, v319, 52.

7. Moriya, H., *Yakugaku Zasshi,* 1959, v79, 1451.

8. Ellis, S., Simpson, M. E., *J. Biol. Chem.,* 1956, v220, 939.

9. Roberts, P. S., *ibid.,* 1958, v232, 285.

10. Frey, E. K., Kraut, H., Werle, E., *Kallikrein (Padutin)*, 1950, Ferdinand Enke Verlag, Stuttgart.

11. Elliot, D. F., Lewis, G. P., Horton, E. W., *Biochem. Biophys. Res. Comm.,* 1960, v3, 87.

12. Seegers, W. H., Laudaburic, R. H., Johnson, J. F., *Science,* 1960, v131, 726.

13. Becker, E. L., Wilhelm, D. L., Miles, A. A., *Nature,* 1959, v183, 1264.

14. Milstone, J. H., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 361.

15. Austen, F. K., *Immunology,* 1960, v3, 152.

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Tumors Induced in Hamsters by Injection of Rhesus Monkey Kidney Cell Extracts. (26576)

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Previous experiments have shown that hamsters <1 to 3 days of age are susceptible to tumor induction by the SE polyoma virus, (1) a virus isolated from the mouse. Since a large number of simian viruses have been recovered from monkey kidney tissue cultures (2-7) the present experiments were undertaken to find out if rhesus monkey kidney cell cultures harbor a virus also capable of inducing tumors in hamsters.

Materials and methods. Cell extract inocula. Extracts refer to frozen and ground tissue culture cells that had been concentrated by centrifugation, or tumors frozen and ground and diluted with a small amount of balanced salt solution. Primary rhesus monkey kidney cell cultures prepared in the Tissue Culture Section of the Division of Biologics Standards were originally grown in medium containing 2% calf serum, then washed and nourished with synthetic medium 199(8) to which penicillin G and streptomycin sulphate were added to make 100 units and 100 μg/ml respectively and incubated at 36°C for a period of 2 weeks. The nutrient fluids were changed at the beginning of the period and again at the end of one week. At the end of the second week approximately 90 to 95% of the fluid was decanted and centrifuged. The bottles containing the remaining cell sheets were frozen and thawed until all the cells were loosened from the glass. The cell suspension and the sedimented cell debris were then transferred to a cooled mortar, frozen in dry ice and ground while frozen. After thawing, portions of one lot were filtered through a Corning fine-sintered glass filter. The material was injected subcutaneously into <1 to 3 day old hamsters in 0.2 ml doses. Each cell extract was derived from cultures made from pooled cells of 8 to 32 pairs of monkey kidneys. Similar extracts were prepared from human or feline tumors or human cell lines nourished with a defined medium containing 5% horse serum.

Hamsters. Hamsters (*Cricetus auratus*) were raised in the Animal Production Section of Nat. Inst. of Health and were kept in an area free of animals infected with the SE polyoma virus. The animals were injected when <1 to 3 days of age with 0.2 ml doses of cell extracts, cells or tissue culture fluids and cared for in the same manner as the hamsters injected with the SE polyoma virus(1).

The tissues of animals that contained tumors were fixed, either in sodium acetate buffered formalin or in sodium acetate buffered mercuric chloride formalin ("B5")(9), embedded, sectioned and stained with hematoxylin and eosin or with Masson's trichrome technic(10) or Bielschowski's method for reticulum (Foot's modification)(11).

Transplantation experiments. Portions of hamster tumors were removed aseptically and placed in Earle's balanced salt solution (12) containing twice the amounts of penicillin G and streptomycin sulphate used in the culture medium. After 30 minutes at 20°C the tissue was minced finely with scissors and injected subcutaneously into <1 or 3-day-old hamsters.

Tissue cultures for culturing hamster tumor material. Three kinds of cell cultures were employed in an attempt to propagate a virus from the hamster tumors: 1) a continuous line of rhesus monkey kidney cells obtained from Dr. Virginia Evans and maintained with synthetic medium 109(13), 2) primary mouse embryo cell cultures(14) and 3) primary vervet monkey kidney cultures prepared in the Tissue Culture Section by Younger's trypsinization method(15).

Extracts of tumor tissue or minced tumor tissue from hamsters were added to the cell cultures and these were incubated at 36°C and examined weekly for cytopathogenic changes. The nutrient fluids were changed at weekly intervals and passages were made to new cell cultures at 2 to 7 weeks.

Hemagglutination and hemagglutination-inhibition tests. To doubling dilutions of virus or tissue extracts an equal volume of 0.5 ml of 0.4% washed guinea pig erythrocytes in 0.85% sodium chloride solution were added and the mixtures were incubated at 4°C until the cells settled in patterns on the bottom of the tubes as previously described (16). Subcutaneous tumor tissue, liver, and kidney tissue of a given hamster injected with monkey kidney cell extract were each prepared for use in the hemagglutination test by grinding a known weight of tissue with alundum, diluting to make a 20% suspension and centrifugating at 4000 rpm for 5 minutes in an angle head centrifuge to remove the larger particles of tissue. Blood for the hemagglutination-inhibition tests was collected by cardiac puncture of the hamsters and from the femoral vein of monkeys.

Results. Incidence of tumors. Two hun-

TABLE I. Tumors Induced in Hamsters Injected When <1 to 3 Days of Age with Cell Extracts of Different Origins.

Cells origin	Lot	Hamsters inj. No.	Hamsters inj. Date	Total No. of animals surviving without tumors	Days of observation of survivors†	No. of animals with tumors at: Mo 5	7	9	>9
Human tumor	A	11	6/ 2/59	5	473	0	0	0	0
Idem	B	11	6/11/59	9	494	0	0	0	0
Monkey kidney	1	10	6/11/59	1	500	5	8	8	9
Idem	2	13	6/11/59	2	500	3	7	10	11
Cat on human cell lines	X	6	6/19/59	4	293	0	0	0	0
Human tumor	C	9	7/ 6/59	9	459	0	0	0	0
Monkey kidney	3	6	7/10/59	0	301	1	4	4	5
Idem	4	12	7/10/59	1	472	2	7	8	9
"	5	11	11/ 6/59	7	352	0	2	2	4
"	6	7	12/ 1/59	1	327	1	4	6	6
"	7	7	12/ 2/59	6	325	0	0	0	0
"	8	1	12/ 2/59	1	325	0	0	0	0
Human tumor	D	9	12/ 3/59	6	319	0	0	0	0
Monkey kidney	9	12	12/17/59	12	311	0	0	0	0
Idem	10	9	12/31/59	0	207	1	7	7	7
"	11	43*	1/14/60	2	283	20	40	41	41
"	12	23	1/21/60	4	349	4	7	12	17
Human tumor cell line	E	19	2/ 4/60	18	277	0	0	0	0
		219‡		88		37	86	98	109

* Fourteen of these animals were inj. with monkey kidney cell extract that had been filtered through a fine sintered glass filter.

† Maximum time of observation.

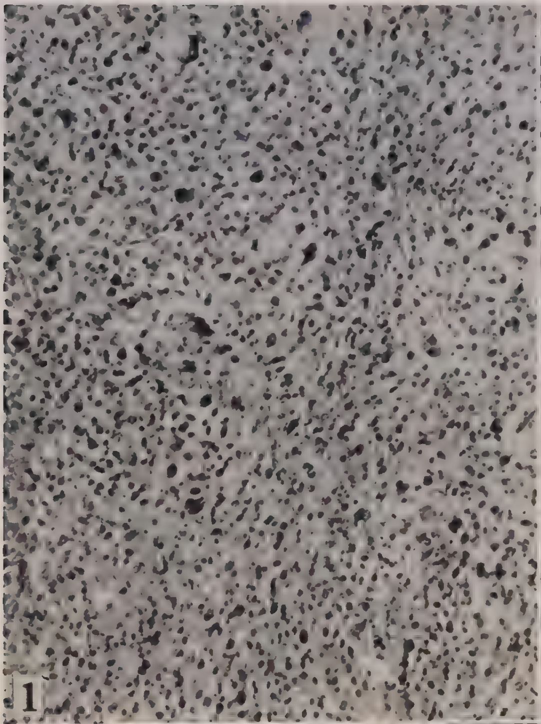
‡ Twenty-two hamsters were lost to the experiment due to early deaths.

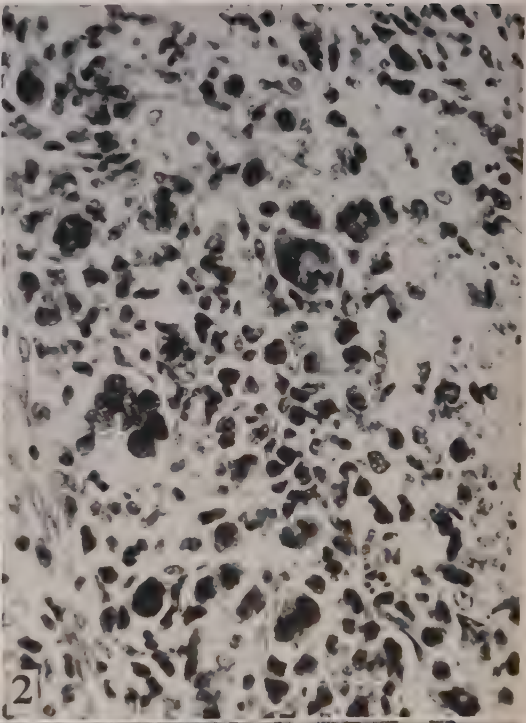
dred and nineteen hamsters were injected with cell extracts of different origins as listed in Table I. One hundred and nine of these animals developed tumors at site of inoculation. Some hamsters were sacrificed for tissue culture or for histological examination; all others with tumors died. All the tumor-bearing animals had received extracts prepared from monkey kidney cell cultures. Of the total of 154 which received monkey kidney cell extracts 70% developed tumors. Fourteen of the hamsters had been injected with filtered monkey kidney cell extracts and 13 of these developed neoplasms which were of the same nature as those that were induced by the unfiltered cell extracts. The earliest tumor was detected 99 days after injection of the cell extract but most of the tumors developed after 7 to 9 months.

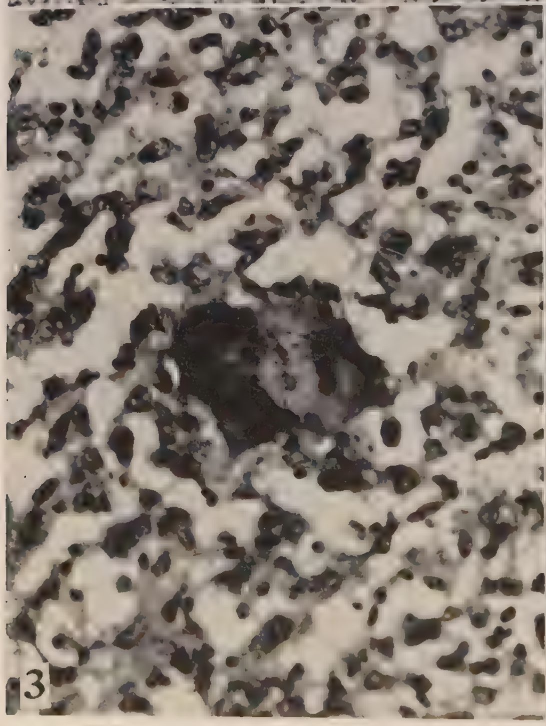
Three monkey kidney cell lots induced no tumors in 19 of 20 hamsters. One of these died from other causes before the experiment was ended. One cell lot, No. 8, was represented by only one hamster.

Transplantation of tumors. Transplants were made to newborn hamsters from 3 of the induced hamster tumors. Transplants from the first tumor injected into 12 hamsters resulted in formation of tumors in 10. These tumors appeared within 13 to 41 days and all 10 hamsters died by the 73rd day. Transplants from the second tumor gave rise to tumors in all of 28 hamsters injected between 14 and 24 days and all were dead on the 45th day. A total of 5 successive transplants were made in hamsters with cells from the second induced hamster tumor and tumors developed in all the surviving animals in from 6 to 24 days. The third induced tumor was transplanted in 12 hamsters and tumors arose in all 12 of these animals in 29 to 48 days.

Pathology. Fifteen hamsters with subcutaneous tumors were examined in detail. The growths were elevated, rounded, and usually firm. On cut surface, these masses were lobulated by invaginations of the fibrous capsule. The underlying skeletal muscle was







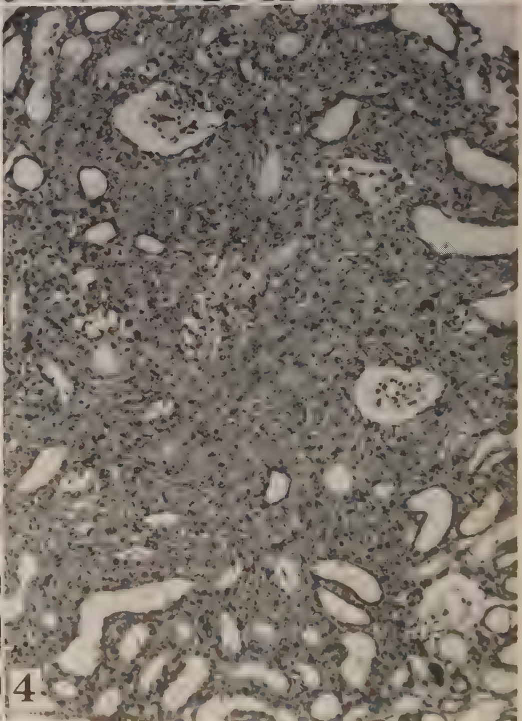


FIG. 1. Undifferentiated sarcomatous cells, some multinucleated in a section of a subcutaneous tumor in a hamster sacrificed at 223 days. H & E. Mag. 100 ×.

FIG. 2. Multinucleated giant cells within subcutaneous sarcoma. H & E Mag. 215 ×.

FIG. 3. A giant cell with basophilic nuclei in subcutaneous tissue in hamster that died at 173 days. H & E Mag. 450 ×.

FIG. 4. Rounded, vesiculated nuclei of tumor cells surrounding tubules and glomeruli in pole of kidney, in hamster sacrificed at 48 days after subcutaneous transplantation. H & E Mag. 100 ×.

TABLE II. Attempts to Culture a Virus from Tumors in Hamsters Induced with Extracts of Monkey Kidney Cells.

Origin of tumor induced by monkey kidney lot No.	Tumor No.	Tissue culture cells	No. of passages	Results
1	A 1*	Monkey kidney stable†	6	No cytopathogenic changes; no tumors developed in 2 litters of hamsters inj. with 2nd and 5th passage fluids and kept for 300 days
1	A 22*	*Idem*	4	No cytopathogenic changes
2	A 25*	"	5	*Idem*
4	A 45*	"	4	"
2	A 46*	Mouse embryo	8	"
		Monkey kidney stable	8	"
2	A 61*	*Idem*	2	"
		Mouse embryo	5	"
9	A 80	Monkey kidney stable	4	"
		Vervet monkey kidney primary	2	Cytopathogenic changes but not different from control cultures
9	A 115‡	Mouse embryo	2	No cytopathogenic changes
		Vervet monkey kidney primary	2	Cytopathogenic changes but not different from control culture
9	A 129	Mouse embryo	5	No cytopathogenic changes

* Hamsters <1 to 3 days of age inj. with the same tumor extract that was added to cell cultures failed to develop tumors when kept for 200 to 300 days.

† The monkey kidney stable cell line originated from rhesus monkey kidney cells.

‡ Cells from minced tumor A 115 were successfully transplanted to other hamsters.

occasionally infiltrated by tumor and the muscle fibers appeared to be engulfed by the tumor cells.

Three hamsters had tumors in the lungs and 2 had similar masses in the kidney.

The subcutaneous tumors consisted mainly of cells with pleomorphic, anaplastic nuclei, and variable amounts of cytoplasm (Fig. 1).

The nuclei were rounded or ovoid, usually vesiculated, with chromatin arranged as in the spokes of a wheel and/or marginated at the perimeter of the nucleus. In some tumors, there were spindle shaped cells and nuclei arranged in interlacing and whorling bundles or in a loose matrix with sparse amounts of cytoplasm. Numerous multinucleated giant cells were present in the sumors (Fig. 2). Many of these were clearly delineated with distinct rounded basophilic nuclei arranged closely within the cell. Others contained fragmented, basophilic nuclear debris within abundant eosinophilic cytoplasm (Fig. 3).

The central portions of the subcutaneous tumors frequently were necrotic. There were many congested and occasionally thrombosed blood vessels within the growths.

The tumors in the lungs and kidneys had a similar histologic pattern as described above (Fig. 4).

The neoplasms appeared to be undifferentiated sarcomas in subcutaneous tissue, kidneys and lungs. Infrequently, there were subcutaneous lobules with histological features resembling fibrosarcomas.

Virologic studies. As shown in Table II 3 kinds of tissue culture were incubated with material from the hamster tumors. No degenerative changes that could be attributed to a virus were observed and the fluids from the second and fifth tissue culture passage of one tumor did not induce tumors in 2 litters of hamsters that were inoculated and kept for 300 days.

Portions of 6 of the tissue extracts that were added to the tissue cultures were injected into litters of newborn hamsters and kept for 200 to 300 days. No tumors were observed in any of these animals.

Tumor No. A 115, was the first tumor transplanted to other hamsters. As mentioned above, tumors developed in 10 of the 12 animals injected.

Portions of the liver, kidneys and tumor tissue of one tumor-bearing hamster were ground separately, diluted in 2-fold steps and incubated at 4°C with 0.4% washed guinea pig erythrocytes. No hemagglutination was noted.

Eighteen of the tumor-bearing animals were bled and their sera tested for hemagglutination-inhibition antibodies against 2 strains of SE polyoma virus. Inhibition did not occur.

Sera were not obtained from the monkeys at the time cell cultures were prepared but sera from 100 normal monkeys from the same colony, tested for hemagglutination-inhibition antibodies against the SE polyoma virus were negative.

Discussion. The SE polyoma virus is the only biological material, other than certain of the monkey kidney cell extracts mentioned in this report, that is capable of inducing tumors in all or almost all hamsters in a litter when injected as newborn. Histologically there are similarities between the present tumors and those induced by the polyoma virus.

The characteristics of lobulation, giant cell reactions, polygonal anaplastic cells, sarcomatous cells with a tendency to differentiate into fibrous tissue were all similar to those in tumors produced by subcutaneous injection of the SE polyoma virus in newborn guinea pigs(17) and hamsters.

The tumors of the present hamsters differed from the polyoma-virus-induced neoplasms in their distribution and cellular detail. No tumors were found in heart, stomach, intestines or liver as occur usually in hamsters inoculated with high titer polyoma virus. The larger number of bizarre giant cells and lack of frequent cellular differentiation of the tumor cells also helped to distinguish these tumors from those produced in response to the polyoma virus. The SE polyoma virus can be propagated in mouse embryo cell cultures inoculated with virus-infected hamster tissue, particularly after one or 2 passages(18) and characteristic cytopathogenic changes occur in the cells(14). Thus far, there has been no evidence that a virus multiplied in the tissue culture cells inoculated with the hamster neoplasms induced with monkey kidney extracts. If a virus in the monkey kidney cell extracts is responsible for the hamster tumors then it is not detectable in the induced hamster tumors. Newborn hamsters did not develop tumors after injection with hamster-tumor extracts. Extracts of polyoma-virus-induced tumors injected directly into other hamsters do not induce tumors(1). To demonstrate polyoma virus in hamster tumors it is necessary to increase the concentration of virus and to free it from antibody by again cultivating the virus in a suitable cell culture(18). Failure to propagate a virus in certain tissue culture lines suggests that at present the tumor-inducing material must be labeled a "substance." Extracts prepared either from human or feline tumor tissue or human tissue cultures failed to induce tumors when inoculated into newborn hamsters.

Summary. Injection of newborn hamsters with extracts of certain lots of rhesus monkey kidney cell cultures has been followed some months later by occurrence of neoplasms at site of injection in 109 of 154 hamsters. Three of the neoplasms were transplanted to other hamsters and the recipient hamsters developed tumors in 13 to 48 days. Transplants from the second tumor were passed from hamster to hamster 5 times and tumors developed in all surviving animals in 6 to 24 days. Tumors could not be induced in newborn hamsters by inoculation of extracts of the hamster tumors. No changes indicative of a virus were observed when extracts of a tumor or a tumor mince were incubated with primary mouse embryo, primary vervet monkey kidney cells, or a continuous line of rhesus monkey kidney cell cultures. The tumors appeared to be somewhat different from those induced by the SE polyoma virus.

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1. Eddy, B. E., Stewart, S. E., Young, R., Mider, G. B., *J. Nat. Cancer Inst.*, 1958, v20, 747.

2. Rustigian, R., Johnston, P., Reihart, H., PROC.

SOC. EXP. BIOL. AND MED., 1955, v88, 8.

3. Hull, R. N., Minner, J. R., *Am. J. Hyg.*, 1956, v63, 204.

4. ———, *Ann. N. Y. Acad. Sci.*, 1957, v67, 413.

5. Mahlerbe, H., Harwin, R., *Brit. J. Exp. Path.*, 1957, v68, 539.

6. Hull, R. N., Minner, J. R., Mascoli, C. C., *Am. J. Hyg.* 1958, v68, 31.

7. Sweet, B. H., Hilleman, M. R., *Internat. Conference on Live Poliovirus Vaccines.* Pan American Health Organization, and World Health Organization, 1960, v2, 79.

8. Morgan, J. F., Morton, J. H., Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 1.

9. Lillie, R. D., *Histopathologic Technic and Practical Histochemistry,* 2nd ed. The Blakiston Co., New York, 1954, p41.

10. Mallory, F. B., *Pathological Technique,* W. B. Saunders Co., Philadelphia 1942, p156.

11. ———, *ibid.,* 161.

12. Earle, W. R., *J. Nat. Cancer Inst.,* 1943, v4, 165.

13. Evans, V. J., Fioramonti, M. C., Sanford, K. K., Earle, W. R., Westfall, B. B., *Am. J. Hyg.,* 1958, v66, 66.

14. Eddy, B. E., Stewart, S. E., Berkeley, W., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 848.

15. Youngner, J. S., *ibid.,* 1954, v85, 202.

16. Eddy, B. E., Rowe, W. P., Hartley, J. W., Stewart, S. E., Huebner, R. J., *Virology,* 1958, v6, 290.

17. Eddy, B. E., Borman, G. S., Kirschstein, R. L., Touchette, R. H., *J. Infect. Dis.,* 1960, v107, 361.

18. Eddy, B. E., Stewart, S. E., *Canadian Cancer Conference,* Academic Press, New York, 1959, v3, 307.

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Significance of Plasma Glycolipid Levels in Normals and in 3 Disorders of Brain Glycolipids.* (26577)

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In 2 neurological disorders of myelin, the kidney may be involved as well as the brain. In the metachromatic form of diffuse cerebral sclerosis, sulfated glycolipids are increased in cerebral white matter, kidney tubules, and urine sediment(1 - 3). In one recent case of the globoid form of diffuse cerebral sclerosis, some kidney epithelial cells have contained unusual material, the staining of which resembles that seen in the diseased white matter(3). Sugar determinations in a heterogeneous lipid extract from one earlier case of this globoid form suggested the possibility that glycolipids might be increased in the brain(4). Recent chemical and direct experimental evidence has suggested the possibility that it may be glycolipids more in the "true" cerebroside category which increase locally in certain globoid cases(3,5). It is the cerebron-like "true" cerebrosides which cause a globoid-like response when injected into rat white matter, whereas thus far injections of other glycolipids (such as sulfatides or gangliosides) have not caused this response (3,5).

If organs as remote as brain and kidney are both involved in some type of glycolipid disorder, 2 major questions arise: (1) Does the material in the kidney merely reflect concentration by the kidney of that material from the diseased brain which has "overflowed" into the circulating plasma? (2) Or, is the material in the kidney largely independent of the plasma and simply an expression in the kidney of the same type of metabolic lesion which occurs, separately, in the brain? One important differentiating point would be the plasma glycolipid level.

The following initial study of plasma glycolipids is reported here, both because these problems of lipid deposits are becoming of wider general biological and conceptual significance and because a most unusual opportunity was presented to study simultaneously a selected series of 4 living patients with 3 rare genetically-determined diseases, the causes of which are still obscure(3).

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Material and methods. Identifying information and biopsy findings on the 2 sibling patients with metachromatic leucoencephalopathy[†] and on the one patient with a globoid form of diffuse sclerosis (Krabbe's disease)[‡] are published elsewhere(3,6-8). Nonhemolysed plasma from the normal male and female control children of comparable ages and from the abnormal control with gargoylism[§] was drawn at comparable times. Plasmas were separated and promptly frozen at –30°C. Control samples were then analysed at the same time, and in the same series with the patient material. The method of Svennerholm(9) was used, with exceptions noted below. The primary extraction of the dried, lyophilized plasma was done in 4 stages with standardized stirring at room temperature(3). A (larger) total of 80 volumes of chloroform-methanol (2:1, v/v) was used per 10 ml of original plasma. After centrifugation at 4000 rpm for 20 minutes and filtration through sharkskin paper, this first lipid extract was dried in a vacuum dessicator. This lipid residue was then reextracted with a total of 16 ml. of solvent and was again filtered. It is emphasized here that water-soluble materials were then removed from this lipid extract by 4 separate washes as indicated (9), but performed in this instance with the aid of a stronger (730 mg %) NaCl solution. The proportion used for the first wash and for the equilibrated wash solution was one volume of NaCl solution to 5 volumes of chloroform-methanol solution. Lipid methods used here have been previously tested in our laboratory and are the same as those cited elsewhere(2). Two separate glycolipid determinations were made at separate times on each purified lipid extract and the results averaged. Deviation from the mean was ± 10.3%. All lipid values are expressed in mg per 100 ml of the original plasma before this plasma was diluted with citrate solution. In Fig. 1, the total glycolipid values have been multiplied by 10 so that they will stand out more easily for comparison among the other lipids in the profile. Dots stand for separate patients or separate determinations; bars stand for means.

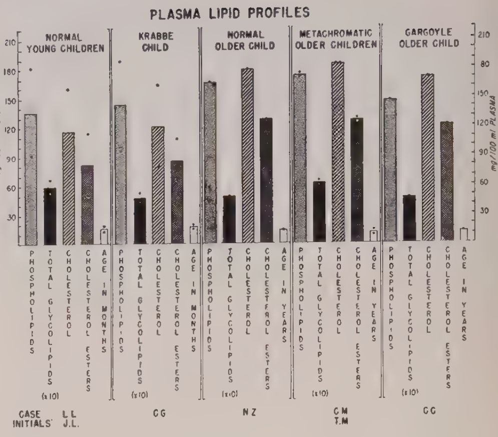

FIG. 1.

Results. With the technic used in this study, glycolipid values for normal children, age 1-12 years, ranged from 4.9 to 6.5 mg %. In Fig. 1, results for normal young children (12-19 mo.) are separated from the normal older child (12 yr.) so that controls of appropriate age may be compared with the patient material.

In the 2 metachromatic patients, the plasma glycolipid values of 6 and 6.3 mg % were only slightly higher than in their control group of comparably older children (4.6-4.9 mg %). In view of the fact that these 2 metachromatic patients are siblings, it is of interest that they show a close correlation in their values, not only in their glycolipid results, but throughout the other determinations in the profile.

The young child with Krabbe's disease (globoid form) shows *no* elevation of plasma glycolipids (4.3-5.1 mg %). It is uncertain whether her somewhat lower values for other lipids at age 15 mo. are to be related to a period of several previous weeks of undernutrition and pneumonitis. This is because her controls show a similar range of variations, and because some serum lipids (*e.g.*: cholesterol) are known to show day to day fluctuations(16). A dilution artifact is not excluded.

[†] Age at onset and age at time of test: C.M. 18 mo. and 12 yr. (autopsy verified); T.M. 8 mo. and 9 yr.

[‡] C. G. 4 mos. and 2 samples at 15 mo. and 18 mo. respectively.

[§] C. C. 1 year and 12 yr. Classical, retarded male gargoyle without cloudy corneae.

Two separate samples of plasma were available from the 2 normal young children, from the one globoid child, and from the 2 metachromatic children. In each patient, the lower of each of these 2 glycolipid values happens to correlate with the lower of the 2 values for phospholipids, total cholesterol, or cholesterol esters, respectively.

Discussion. In the 2 older metachromatic patients, as the neurological and kidney disorder is far advanced, it is questionable whether their slightly higher plasma glycolipid values reflect a plasma trend of primary significance. For example, some general tendency has been noted for certain *non*-glycolipids in blood to be raised nonspecifically in children who have a wide variety of neurological diseases other than those studied here(10). In addition, glycolipid results in 3 younger metachromatic children have appeared since completion of the present study. In these patients, the glycolipid values (3.4-3.9 mg %) fell within the range of the normals (2.7-4.7 mg %)(13).

Even the presence of an early, gross and consistent elevation in a certain plasma glycolipid fraction would leave a problem unsettled. Specifically, such an elevation need not only reflect material actually enroute from degenerating brain; it might rather represent material first synthesized in the kidney (or some other systemic organ) which then spilled out into the plasma. However, a *normal* plasma glycolipid value may be one point more in favor of an independent origin of a metabolic abnormality in brain and kidney. Of particular interest in this regard is the normal trend in plasma glycolipids of the living globoid (Krabbe) child. Viewed together with the small absolute amounts of glycolipids which we have been able to extract from devastated white matter of 2 other globoid patients(3,5), these results would not suggest one disease which has a necessarily simple process of organ storage and plasma overflow. If such exists, it would not appear to be readily definable by these methods. Moreover, with plasma glycolipid values if anything slightly toward the low normal side, the potential mechanism in storage diseases of an increased binding of tissue glycolipids to *other* moieties in tissues still remains to be considered. Because the metachromatic and globoid categories of diseases may each include subtly different disease mechanisms(3,5), it follows that in future series of cases each patient must still be individualized.

This lack of elevation in plasma from CG is of further interest because of the recent report of an *increase* of plasma glycolipids in (splenectomized) children with Gaucher's disease—a disorder in which a glycolipid storage in visceral organs is established(11). Hence, the old issue of what role the plasma lipids play in the visceral deposits in an *individual* Gaucher patient is again reopened, because Thannhauser found *normal* glycolipid values in (non-splenectomized) adult Gaucher patients(12).

Nothing is known about the turnover rate of the plasma glycolipid compartment *per se*, or of the equilibrium it shares with various organ systems in health or disease. However, for the brains of infants and young children to grow rapidly, it is plausible that certain brain glycolipids are in a more dynamic relationship with their plasma precursors and plasma counterparts (at least when contrasted with the adult). Moreover, despite the fact that the brain and the plasma compartments are several steps removed metabolically, and are separated by a "blood-brain" barrier in the adult, there may still be a significant degree of *internal* turnover within these brain glycolipids themselves(14,15). A tendency in the present series for a lower glycolipid value to correlate with lower values for other lipids from the same patient is more likely fortuitous than a reflection of a real metabolic interrelationship.

Glycolipid results here, as well as those of Svennerholm, and the still lower values of Thannhauser, are lower than the "cerebroside" results reported earlier, presumably in part because any water-soluble glyco-complexes|| (which might be extracted) would

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|| e.g.: mucopolysaccharides, gangliosides, etc.

now be removed during the washing steps(9). The classical gargoyle was originally included in this series, both as a possible test of this very point and as an appropriate control for the 2 authentic metachromatic patients who also have these gargoyle-like laboratory findings. However, in the first lipid extract of gargoyle plasma, a moderately turbid precipitate developed, some of which then adhered to the glassware. This precipitated material was no longer soluble at room temperature in a chloroform-methanol solution. Lipid results recorded for all patients here clearly refer to the usual extractable and continuously *soluble* lipids, and are not intended to include a less conventional plasma fraction which would be removed by precipitation and filtration (as was this gargoyle fraction, for example).

The small size and terminal condition of our patients limits the volume of plasma which can be safely withdrawn for analyses. Hence, here as elsewhere(13), there is a corresponding limitation on our knowledge of exactly how much of the total plasma glycolipids may exist in the form of sulfatides, and how much is in the form of *non*-sulfated glycolipids.

Summary. 1. Plasma glycolipids and other plasma lipids were studied in 2 diseases affecting the myelin glycolipids, in gargoylism, and in controls of appropriate age. 2. Plasma glycolipid levels in this series of normal children ranged from 4.9-6.5 mg %. Those in 2 metachromatic children ranged from 6.0-6.3 mg %. In the globoid child, plasma glycolipids ranged from 4.3-5.1 mg %. 3. Data obtained with these methods would not appear to establish a disorder of total plasma glycolipids *per se* as the primary source for systemic deposits in the patients studied. 4) The significance of the plasma glycolipid level is considered, and the need for individualization in further studies is stressed.

It is a pleasure to acknowledge the assistance of the Dept. of Pediatrics and of Mrs. Margaret Bischel in obtaining plasma samples, and that of Mrs. Jean Scott and Mr. David Gaudin in cholesterol and phosphorous determinations.

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1. Austin, J., Proc. Soc. Exp. Biol. and Med., 1959, v100, 361.

2. ———, *Neurology,* 1960, v10, 470.

3. ———, *Assn. Res. Nerv. Ment. Dis.,* Williams & Wilkins Co., Baltimore, Dec. 1960, v40, in press.

4. Cumings, J., *Brain,* 1955, v78, 554.

5. Austin, J., Lehfeldt, D., Maxwell, W., Am. Assn. Neuropath., June 1960; *J. Neuropath. and Exp Neurol.,* 1961, v20, 284.

6. Austin, J., *Neurology,* 1957, v7, 415.

7. ———, *ibid.,* 1957, v7, 716.

8. ———, *Tr. Am. Neurol. Assn.,* 1958, 149.

9. Svennerholm, E., Svennerholm, L., *Scand. J. Clin. & Lab. Invest.,* 1958, v10, 97.

10. Terslev, E., *Acta Paed.,* 1960, v49, 69.

11. Hillborg, P., Svennerholm, L., *ibid.,* 1960, v49, 707.

12. Thannhauser, S., *Lipidoses, Diseases of the Intracellular Lipid Metabolism.* 3rd ed., Grune & Stratton, N. Y., 1958.

13. Hagberg, B., Svennerhclm, L., *Acta Paed.,* 1960, v49, 690.

14. Radin, N., Martin, F., Brown, J., *J. Biol. Chem.,* 1957, v224, 499.

15. Moser, H., Karnovsky, M., *ibid.,* 1959, v234, 1990.

16. Peterson, J., Wilcox, A., Haley, M., Keith, R., *Circ.,* 1960, v22, 247.

Received March 13, 1961. P.S.E.B.M., 1961, v107.

Experimental Pyelonephritis in Rats with Aminonucleoside-Induced Nephrotic Syndrome.* (26578)

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(Introduced by Henry D. Moon)
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The normal kidney is usually quite resistant to infection by organisms known to cause pyelonephritis. The susceptibility of the kidney to experimental infection, however, is increased by injuries such as induced ureteral obstruction(1,2), renal massage(3), scar formation(4), lesions of potassium depletion(5) and treatment with desoxycorticosterone acetate-saline(6). Clinically, urinary tract obstruction has long been recognized as predisposing to pyelonephritis and various other predisposing factors have been suspected.

The clinical observation of a high incidence of urinary tract infections in patients with the nephrotic syndrome(7) prompted us to investigate the susceptibility to pyelonephritis in rats with experimentally induced nephrosis. Many of the methods for production of pyelonephritis employ extrarenal or intrarenal urinary obstruction as the predisposing factor(8). Recent studies, however, have shown that a nephrotic syndrome can be induced in experimental animals by administration of the aminonucleoside, 6-dimethyl-aminopurine 3-amino-d-ribose (AMN) (9), and that the renal changes, as seen by light and electron microscopy(10), closely resemble those found in many cases of nephrosis in humans. In an attempt to clarify factors other than obstruction that might predispose the kidney to infection, rats pretreated with AMN were used in the present study. After induction of bacteremia, incidence of pyelonephritis and rate of disappearance of the injected organisms from the kidneys was determined by histologic examination and quantitative bacteriologic culture of renal tissue from experimental and control animals.

Materials and methods. Long-Evans rats weighing 150 to 200 g were used. Nephrosis was induced by subcutaneous injection on 2 consecutive days of a 0.5% aqueous solution of AMN in doses of 0.3 ml per 100 g of body weight. An 18-hour broth culture of *E. coli*,† containing approximately 10^8 organisms per ml, was administered in a dose of 0.5 ml into the jugular vein.

The rats were divided into 4 groups. Group I consisted of 12 normal, untreated animals. The remaining 3 groups were comprised of 60 animals each. The rats in Group II were injected with *E. coli* and sacrificed in sets of twelve 2, 7, 14, 21 and 30 days after injection. The animals in Groups III and IV were treated with AMN. Urinary protein excretion was measured daily by the sulfosalicylic acid method. The rats developed 4+ proteinuria by the 7th to 10th day after injection of AMN. The rats in Group III were sacrificed in sets of twelve 2, 7, 14, 21 and 30 days after appearance of 4+ proteinuria. The rats in Group IV were injected with *E. coli* as soon as they developed 4+ proteinuria, and were sacrificed in sets of twelve 2, 7, 14, 21 and 30 days after injection.

The rats were killed with ether and autopsied immediately. The kidneys were removed under sterile conditions, and each was cut into 6 slices. Alternating slices were used for quantitative bacterial cultures and for microscopic sections stained with hematoxylin and eosin.

Bacterial cultures were prepared by grinding 0.5 to 1.2 g of kidney in a sterile mortar and adding broth (2 ml/g). Serial dilutions were prepared, plated on blood agar and incubated for 24 hours. Bacterial colonies were

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† Gray strain, obtained from Dr. A. I. Braude, Univ. of Pittsburgh School of Medicine, Pittsburgh, Pa.

TABLE I. Incidence of Pyelonephritic Lesions and Presence of Coliform Organisms per Rat Kidney.

	Histologic examination			Bacteriologic examination					
					E. coli/g kidney				
Group & interval	Neg.	"Non-specific"	Pyelo-nephritis	Neg.	$<10^3$	10^3-10^4	10^4-10^5	$>10^5$	Significant non-coliform organisms
I (normal control)	21	3	0	24					
2 days									
II (*E. coli*)	9	15	0	0	0	5	10	9	0
III (AMN)	15	9	0	24	0	0	0	0	0
IV (AMN & *E. coli*)	4	20	0	0	0	8	4	12	0
7 days									
II	16	8	0	4	15	1	1	3	0
III	13	11	0	24	0	0	0	0	0
IV	0	21	3	6	3	4	3	7	1
14 days									
II	10	13	1	14	9	0	0	0	1
III	17	6	1	24	0	0	0	0	0
IV	1	12	11	13	8	1	0	2	0
21 days									
II	23	1	0	19	4	1	0	0	0
III	16	8	0	24	0	0	0	0	0
IV	3	14	7	19	3	0	0	2	0
30 days									
II	18	5	1	24	0	0	0	0	0
III	20	4	0	24	0	0	0	0	0
IV	5	8	11	17	3	1	0	3	0

then counted, and number of organisms per g of renal tissue was calculated. All coliform organisms were subjected to antibiotic sensitivity tests to determine whether they were identical with the injected strain. Occasional cultures contained small numbers (usually less than 20 per g of tissue) of organisms such as nonhemolytic *Staphylococcus albus* or *Alpha streptococcus,* which were considered to be contaminants and were disregarded.

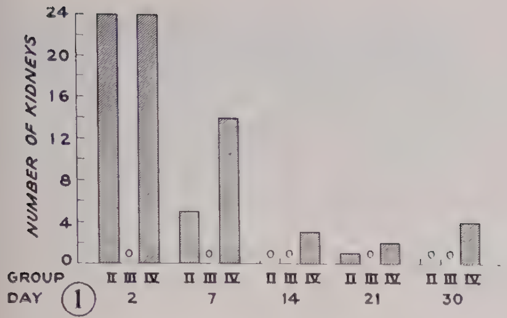
Histologic criteria. Lesions were considered to be pyelonephritic when inflammatory cells, consisting of lymphocytes and varying numbers of polymorphonuclear leukocytes and macrophages, extended in sheets between the renal tubules, and were also present to a variable degree within the tubules and the renal pelvis. Older lesions contained occasional plasma cells.

Infiltrates were considered "nonspecific" for any disease process when they consisted of small numbers of cells, mostly lymphocytes, located within the interstitial tissues but not within the tubules and pelvis.

Results and discussion. The results of serial histologic and bacteriologic examinations in the 4 groups of animals are summarized in Table I and graphically presented in Fig. 1 and 2.

The normal control rats (Group I) showed no evidence of pyelonephritis. A "nonspecific" infiltrate was observed microscopically in 3 kidneys, but no *E. coli* were demonstrable by quantitative cultures.

The rats injected with AMN alone (Group III) developed degenerative and regenerative changes of the renal tubules. Hyaline casts were frequent, and in some cases small numbers of chronic inflammatory cells were seen surrounding the affected tubules (Fig. 3). In this group, only one kidney showed a minute focus of pyelonephritis on microscopic examination. No evidence of pyelonephritis was obtained by bacteriologic cultures. Bacteremias probably occurred in these animals, but evidently not to a sufficient degree to cause pyelonephritis. If the experiment had been carried out for a longer period, the incidence of spontaneous pyelonephritis in this group might have been greater.



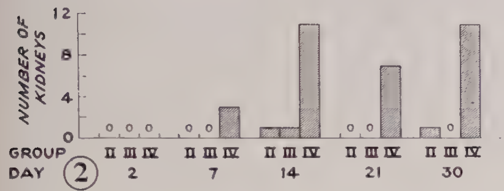


FIG. 1. No. of kidneys with bacterial counts > 10^3 *E. coli*/g of tissue in Groups II, III, IV.

FIG. 2. No. of kidneys with histologic lesions of pyelonephritis in Groups II, III, IV.

The rats with AMN-induced nephrosis (Group IV) showed a significantly increased susceptibility to hematogenous pyelonephritis when challenged with *E. coli,* compared to the rats injected with *E. coli* alone (Group II). The difference between Groups II and IV became evident on histologic examination 7 days after injection of *E. coli,* with occurrence of 3 cases of pyelonephritis in the nephrotic rats and none in the controls injected with *E. coli* only. The difference between Groups II and IV became even more marked with time. At 14, 21 and 30 days after injection of *E. coli,* 11, 7 and 11 cases of pyelonephritis, respectively, had occurred in Group IV, compared to 1, 0 and 1 cases in Group II. Analysis by the Chi-square test, using the Yates correction, showed these differences to be highly significant ($p < .01$).

The lesions seen earlier in the experiment showed an admixture of polymorphonuclear leukocytes and lymphocytes to a variable degree, and occasional lesions consisted almost entirely of polymorphonuclear leukocytes (Fig. 4). Later, lymphocytes dominated the picture, with occasional plasma cells present (Fig. 5). Toward the end of the experiment, the lesions showed a tendency to heal.

The differences between Group II and IV on quantitative bacterial culture were less striking; however the kidneys of the Group IV animals tended to contain larger numbers of organisms than those of Group II. *E. coli* were gradually removed from the normal rat kidney (Group II). Small numbers of organisms were demonstrable by culture up to 21 days after the injection in a few animals, but were no longer detectable at 30 days. In contrast, large numbers of *E. coli* were still demonstrable in 3 kidneys of Group IV animals at 30 days, and small numbers of organisms were observed in 4 others. In all cases the coliform organisms recovered from the kidneys were identical with the injected strain. Significant numbers of organisms other than the injected strain of *E. coli* were found in only 2 kidneys, one from an animal in Group II and one from a animal in Group IV.

The focal nature of pyelonephritis probably accounts for the poor correlation between histologic and bacteriologic findings. The affected kidneys usually contained only one or a few small foci. The lesion, therefore, may have been present in the tissue examined histologically but not in the specimen used for bacterial culture, or vice versa.

"Nonspecific" renal infiltrates were found in all 4 groups (Table I). In the kidneys of rats injected with *E. coli* alone (Group II), the infiltrates consisted of a few polymorphonuclear leukocytes and macrophages within the medullary interstitial tissues near the

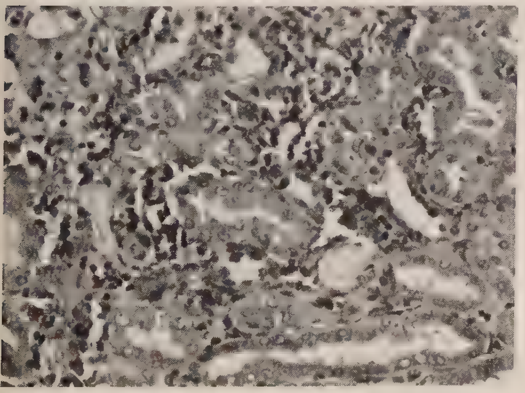
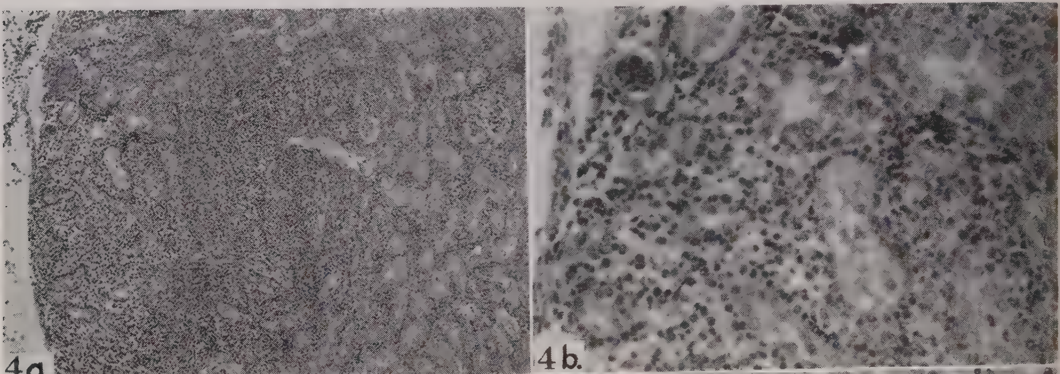
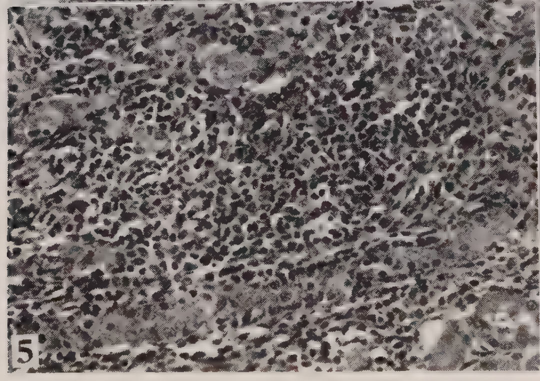


FIG. 3. Kidney of rat from Group III 7 days after onset of 4+ proteinuria, showing a "nonspecific" infiltrate, presence of protein within tubules and degenerative tubular changes. 204 ×.





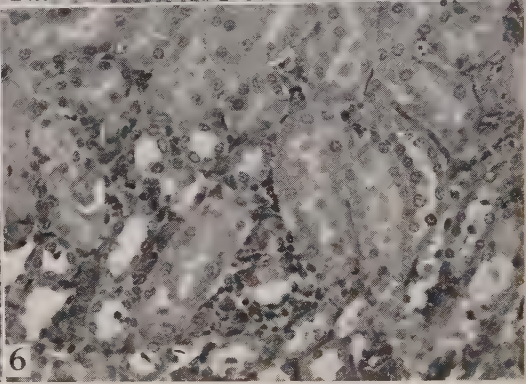


FIG. 4. a. Kidney of rat from Group IV 7 days after injection of *E. coli,* showing a typical cortical pyelonephritic lesion, consisting of a massive interstitial infiltrate of polymorphonuclear leukocytes, lymphocytes and macrophages. A tubule is also filled with inflammatory cells. 75 ×. b. Same lesion at higher magnification. 204 ×.

FIG. 5. Kidney of rat from Group IV 30 days after injection of *E. coli,* showing an older pyelonephritic lesion, consisting of a marked interstitial infiltrate of lymphocytes, macrophages and rare plasma cells. 204 ×.

FIG. 6. Kidney of rat from Group II 7 days after injection of *E. coli,* showing a small "non-specific" infiltrate. 204 ×.

corticomedullary junction (Fig. 6). Although these lesions were probably caused by bacteria, a diagnosis of pyelonephritis could not be made by pathological standards.

The results of this study show that AMN-induced nephrosis predisposes the rat kidney to pyelonephritis. Most of the factors previously shown to increase the susceptibility of the kidney to infection depend to a large extent on intrarenal or extrarenal obstruction of urine flow. In AMN-induced nephrosis, obstruction apparently is not a major predisposing factor to renal infection since the primary anatomic site of injury is in the glomerulus. Although tubular changes are present, the kidneys show no evidence of intrarenal obstruction. Thus, it is more likely that chemical or functional vascular alterations are the important factors in the pathogenesis of this type of pyelonephritis. A recent study of the enzymatic activity in homogenates of kidneys from rats with AMN-induced nephrosis(11) showed a decrease in alkaline phosphatase and an increase in hexokinase and glucose 6-phosphate-dehydrogenase. It remains to be seen whether the increased susceptibility to pyelonephritis in AMN-induced nephrosis can be related to changes in renal enzyme patterns, thus providing a common denominator for the various types of renal injury predisposing to infection.

Summary. Rats with nephrosis induced by treatment with aminonucleoside showed an increased susceptibility to pyelonephritis when challenged with *Escherichia coli.* The incidence of pyelonephritic lesions, determined histologically, was significantly greater

in these animals than in rats injected with *E. coli* only. The organisms were gradually removed from the normal kidney and were no longer demonstrable by culture 30 days after injection. In contrast, at 30 days, significant numbers of *E. coli* were still present in the kidneys of some rats with aminonucleoside-induced nephrosis. It is suggested that functional vascular alterations or, possibly, changes in renal enzyme patterns may be the important factors in the pathogenesis of this type of pyelonephritis.

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1. Guze, L. B., Beeson, P. B., *J. Exp. Med.*, 1956, v104, 803.

2. Lepper, E. H., *J. Path. Bact.*, 1921, v24, 192.

3. Braude, A. I., Shapiro, A. P., Siemienski, J., *J. Clin. Invest.*, 1955, v34, 1489.

4. De Navasguez, S., *J. Path. Bact.*, 1956, v71, 27.

5. Woods, J. W., Welt, L. G., Hollander, W., Jr., Newton, M., *J. Clin. Invest.*, 1960, 39, 28.

6. Woods, J. W., *ibid.*, 1958, v37, 1686.

7. Hopper, J., Jr., Yamauchi, H., personal observation.

8. Kleeman, C. R., Hewitt, W. L., Guze, L. B., *Medicine* (Baltimore) 1960, v39, 3.

9. Fiegelson, E. B., Drake, J. W., Recant, L., *J. Lab. Clin. Med.*, 1957, v50, 437.

10. Vernier, R., Papenmaster, B. W., Good, R. A., *J. Exp. Med.*, 1959, v109, 115.

11. Dubach, U. C., Recant, L., *J. Clin. Invest.*, 1960, v39, 1364.

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Oxidation of Palmitate-1-C^{14} by Red Blood Cells.* (26579)

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It is well established that many body tissues have the enzymatic equipment for oxidation of both glycerides and carbohydrates as a source of energy. Isotope tracer studies have shown that both these processes occur simultaneously in tissue cells, where either glucose or fatty acid oxidation may prevail to a different extent according to the experimental conditions. The non-esterified fatty acids (NEFA) of the blood plasma, present in water-soluble albumin complexes, have been shown to be highly active metabolites(1,2). They appear to be the prerequisite form for penetration of long-chain fatty acids into sites of oxidation(3,4) and the flux of calories carried by NEFA may reach more than twice the caloric value of glucose transport(5), although NEFA comprise only about 5% of the total fatty acids of the plasma.

It has long been known that red blood cells oxidize glucose as a source of energy(6). Although biosynthesis of fatty acids by red blood cells has been investigated by James, *et al.*(7), oxidation of fatty acids by red blood cells, to our knowledge, has not been examined.‡ However, oxidation of palmitate-1-C^{14} by skeletal muscle *in vitro* has been examined by Fritz, *et al.*(8), and its oxidation by hepatic and adipose tissues has been examined by Milstein and Briscol(9) and others. The aim of our investigation was to ascertain the existence of an enzymatic mechanism for fatty acid oxidation in red blood cells.

Methods. Normal, fed, adult, male rats, weighing between 200 and 400 g were killed by decapitation and rapid exsanguination. The blood was collected into about 15 to 20 times its own volume of modified Tyrode phosphate medium, (NaCl—0.137 M; KCl—0.004 M; Na_2HPO_4 buffered to pH 7.38—0.01 M). No anti-coagulants were used because this dilution of blood is sufficient to prevent formation of clots without their use.

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‡ A paper on "Incorporation of palmitate-1-C^{14} incorporation into blood cells" by Mines, C. J., Fillerup, D. L., Mead, J. F., *Nature* 1961, v190, 92, has just appeared as the present paper goes to press.

Red blood cells were washed and centrifuged several times in the modified Tyrode phosphate medium.

Palmitic-1-C^{14} acid (specific activity 1.99 millicuries/millimole) was purchased from Nuclear-Chicago Corp. and stored dissolved in benzene. For each experiment an aliquot of the benzene solution is pipetted into a small volumetric flask, evaporated in a mild stream of air, then neutralized with 0.01 M KOH solution in slight excess and dissolved at 75°C, and, finally, adjusted to the mark with modified Tyrode phosphate medium. Because non-enzymatic evolution of $C^{14}O_2$ from palmitate-1-C^{14} has been shown to be of substantial magnitude(9,10), freshly prepared substrate is used for each experiment.

The incubation of washed red blood cells with palmitate-1-C^{14} is done in Conway microdiffusion units. Into their outer well are added: (1) 0.5 to 2.0 ml of a 1.0 to 10% suspension of washed red blood cells; (2) 0.5 to 1.0 ml of a 0.05 mM solution of palmitate-1-C^{14} in modified Tyrode phosphate medium; (3) plus a sufficient volume of modified Tyrode phosphate medium to adjust the total volume to 4.0 ml.

Into the center well of the Conway microdiffusion unit is inserted a small removable stainless steel cup, containing one ml of 10% KOH solution, to capture the $C^{14}O_2$ evolved during the incubation. After an incubation period of exactly 2 hours at room temperature (about 24°C), the microdiffusion units are opened and the removable cup with the 10% KOH, containing the evolved $C^{14}O_2$, is replaced by another cup with 10% KOH, and the microdiffusion units are sealed again for a new period of incubation. The $C^{14}O_2$ evolved during each incubation period and captured in 10% KOH is precipitated with an excess of 1 M barium chloride and $BaC^{14}O_3$ is recovered and assayed for radioactivity. For each such experimental determination, controls were run in microdiffusion units, with the same amounts of palmitate-1-C^{14}, but with modified Tyrode phosphate medium alone, in substitution for the red blood cell suspension, with final volume of incubation adjusted again to 4.0 ml. Isotope measurements are carried out with the Automatic Scaler Unit, model 161A, from Nuclear-Chicago Corp. (The background is about 16 to 18 counts per minute.) Radioactivity is usually measured as the time for 6400 counts.

TABLE I. Oxidation at Room Temperature of Palmitate-1-C^{14} (0.0125 mM Concentration) by Varying Concentrations of Washed Red Blood Cells.

	Evolved $C^{14}O_2$*		
	Red blood cell concentration		
Time (hr)	1%	2%	4%
0-2	34.5	61.1	124.2
2-4	66.4	99.3	173.4
4-6	36.6	57.4	125.4
6-8	18.2	26.4	39.5

* Counts/min. for $BaC^{14}O_3$ precipitate, representing difference between experimental and blood cell-free control.

Results. After numerous trial experiments for establishment of suitable experimental conditions for oxidation of palmitate-1-C^{14} by red blood cells *in vitro,* many comprehensive experiments of the type shown in Tables I and II were carried out. Data of these tables are typical of those obtained from all these experiments, which have been performed with various amounts of red blood cells, incubated with various concentrations of palmitate-1-C^{14} during the various length of incubation time. At least 8-10 such experiments are represented by each value shown in the tables.

Table I shows that oxidation of palmitate-1-C^{14} is proportional to number of red blood cells, and that oxidation increases with length of incubation time, though not quite proportionally. The amount of the metabolic $C^{14}O_2$ evolved is lower in the first interval of incubation, consistently higher in the second in-

TABLE II. Oxidation at Room Temperature of Varying Concentrations of Palmitate-1-C^{14} by a 4% Suspension of Washed Red Blood Cells.

	Evolved $C^{14}O_2$*				
	Concentrations of palmitate-1-C^{14} in mM				
Time (hr)	.0008	.0016	.0032	.0063	.0125
0-2	18.1	26.4	48.3	77.4	124.2
2-4	23.2	43.6	64.1	114.4	173.4
4-6	10.3	28.5	48.4	69.2	125.4
6-8	6.8	13.7	17.6	23.4	39.5

* Counts/min. for $BaC^{14}O_3$ precipitate, representing difference between experimental and blood cell-free control.

terval, and decreases in the third and fourth interval of incubation.

Table II shows that the amount of evolved $C^{14}O_2$ during oxidation of palmitate-1-C^{14} by a constant number of red blood cells increases with increasing concentration of palmitate-1-C^{14} in the solution. However, for the 16-fold increase in concentration of palmitate, release of the metabolic $C^{14}O_2$ is only about 9 to 12 times higher, which indicates an increasing "saturation" of red blood cells with palmitate as a source of energy, even at comparatively very low concentrations of palmitate. At higher concentrations of palmitate, this saturation becomes even more pronounced.

Discussion. The data clearly demonstrate that red blood cells oxidize palmitic acid to CO_2. However, it is likely that the *in vivo* rate of fatty acid oxidation by red blood cells is considerably reduced by the presence of naturally occurring substances having inhibitory effects. The factors which may influence rate of fatty acid oxidation by red blood cells *in vivo* are largely unknown and are being investigated. Preliminary experiments indicate that their influence increases with age of the animal.

Experiments reported here do not permit inference as to the fate of other carbons in the fatty acid molecule. The generally accepted scheme of fatty acid metabolism suggests that all straight chain fatty acids are activated and then oxidized to acetyl CoA(11). Once a fatty acid begins to be oxidized, it continues to be cleared of acetyl fragments until the whole molecule is degraded(12). The odd-numbered carbons on a long-chain fatty acid share nearly, but not exactly, the same metabolic fate(12).

Since the appearance of $C^{14}O_2$ is the result of a series of enzymatic reactions, it is not possible to give absolute conversion rates of added labeled palmitate to CO_2. Any unlabeled substrate that contributes to the final common acetyl CoA pool would dilute the collected $C^{14}O_2$. The data in Tables I and II demonstrate relative changes in rates of fatty acid oxidation which can be induced by changes in number of red blood cells, in fatty acid concentration, in duration of incubation, and by changes in other experimental conditions, some of which are currently being investigated.

Summary. Washed, red blood cells from normal, fed, adult rats were incubated *in vitro* in a modified Tyrode phosphate medium with palmitate-1-C^{14}, the oxidation of which was demonstrated and measured by following the incorporation of radioactivity in the respiratory CO_2. Rate of palmitate oxidation to CO_2 has been shown to increase with number of red blood cells in the incubating vessels and with concentration of palmitate, and also depends on duration of the incubation. The data are discussed in relation to the present concepts of fat metabolism *in vivo*.

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1. Felts, J. M., *Fed. Proc.*, 1957, v16, 37.
2. McCalla, C., Gates, H. S., Jr., Gordon, R. S., Jr., *Arch. Biochem. Biophys.*, 1957, v71, 346.
3. Gordon, R. S., Jr., *J. Clin. Invest.*, 1957, v36, 810.
4. Gordon, R. S., Jr., Cherkes, A., *ibid.*, 1956, v35, 206.
5. Dole, V. C., *Arch. Int. Med.*, 1958, v101, 1005.
6. Barron, E. S. G., Harrop, G. A., Jr., *J. Biol. Chem.*, 1928, v79, 65.
7. James, A. T., Lovelock, J. E., Webb, J. P. W., in: *Essential Fatty Acids*, edited by H. M. Sinclair, Butterworths Scientific Publications, London, 1957, p72.
8. Fritz, I. B., Davis, D. G., Holtrop, R. H., Dundee, H., *Am. J. Physiol.*, 1958, v194, 379.
9. Milstein, S. W., Driscoll, L. H., *J. Biol. Chem.*, 1959, v234, 19.
10. Artom, C., *ibid.*, 1953, v205, 101.
11. Green, D. E., *Biol. Rev.*, 1954, v29, 330.
12. Chaikoff, I. L., Brown, G. B., in: *Chemical Pathways of Metabolism*, edited by D. M. Greenberg, Acad. Press, N. Y., 1954, vI, p277.

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Experimental Evaluation of a Marlex Vascular Prosthesis.* (26580)

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A variety of vascular prostheses are available, yet search for the ideal graft continues as indications and demands for grafts increase (1). The use of synthetic materials as vessel replacement is progressing and work along these lines must continue as new materials and methods of production are developed. Recently a high density polyethylene called Marlex has been shown to possess ideal physical and biologic properties as a surgical prosthesis and clinical observations proved its usefulness in replacement of tissue defects (2). The present study was undertaken to test the suitability of this plastic as a vascular prosthesis.

Methods. The Marlex used has a tensile strength of 50,000-150,000 lb/sq inch, is nonwettable and is resistant to acids, alkalies and to most organic solvents. It is thermostable at temperatures up to 250°F and sterilization may be accomplished either by boiling or with chemical agents. The tubular prosthesis used was knitted from 2 strands of 70 denier multi-filament yarn to a diameter of 10 mm. The individual prostheses were crimped and cut at lengths from 15 to 25 cm.

The prosthesis was implanted as a long bypass graft between the mid-thoracic and lower abdominal aorta in 22 dogs. The method of bypass was essentially the same as described by McCune and Blades(3). Through a thoraco-abdominal or separate flank and thoracic incisions, the side of the abdominal aorta immediately proximal to the trifurcation was anastomosed to the distal end of the graft. The graft was then tunnelled retro-peritoneally and retro-pleurally through a hiatus made in the posterior attachment of the left leaf of the diaphragm, and an end-to-end anastomosis was performed between the graft and proximal end of the divided thoracic aorta. The distal stump was sutured. This method of vascular bypass insured reliance on a patent prosthesis for survival of the animal.

The quality of the femoral pulses and general condition of the animals were recorded bi-monthly, and the state of the grafts was noted by interval percutaneous transventricular aortography. The animals were sacrificed at 1, 3, 6 and 12 months after operation. An aortogram was made at time of sacrifice and the graft and adjacent aorta were removed and the specimen placed in formalin (10%). After fixation selected specimens were washed and placed in alcohol 80% to regain color and then photographed. Representative portions were embedded in paraffin and consecutive microscopic preparations were stained with hematoxylin and eosin, Masson's trichrome, and Weigert's elastic tissue stains.

Results. Since the graft is of a porous knit, blood loss is considerable at the initial restoration of blood flow; however, the loss is not excessive if care is taken to pre-clot the graft and allow coagulation to occur in the crevices of the fabric by intermittent interruption of flow through the graft. Of the 22 animals in which the prosthesis was inserted as a long aortic bypass 18 survived for 48 hours or longer. Four animals died in the first 48 hours; however, in none was the cause of death related to the prosthesis *per se.* One dog died 3 weeks following operation because of disruption of the proximal anastomosis in an infected wound. Another dog died 3 months after implantation of the graft as the result of malabsorption and it is assumed that there was impairment in the retrograde flow by which the blood from the bypass reaches the abdominal viscera. The remaining 16 animals were sacrificed at various periods. Each maintained normal femoral pulses up to day of death. Interval aortograms demonstrated patency of all

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* This work was supported by grants from Phillips Chemical Co. and U.S.P.H.S.

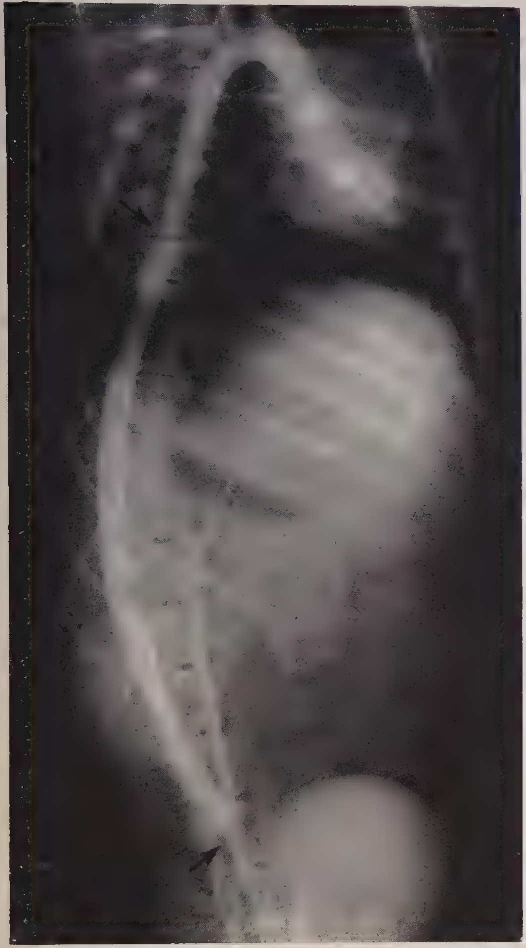

FIG. 1. Angiogram of dog illustrating a Marlex vascular prosthesis in place for 6 mo.

grafts without dilatation or constriction along the bypass or at either anastomosis (Fig. 1). None of the implanted prostheses exhibited stenosis, aneurysm or plaque formation. Four prostheses, however, contained small deposits of fibrin on the luminal surface in areas of kinking. In the rest of the prostheses as well as in those without kinks, there was a smooth glistening lining. The entire intimal surface of the prosthesis had this smooth lining 3 months after implantation. A moderately thick layer of fibrous tissue surrounded the implants and the peri-prosthetic tissue was firmly attached to the interstices of the fabric. Tensile strength of the prosthesis appeared unchanged after one year of implantation.

In Fig. 2 is shown a Marlex prosthesis in place for 9 months. The proximal segment of the aorta is continuous with a Marlex bypass prosthesis 18 cm long anastomosed to distal abdominal aorta. The prosthesis is encased in fibrous connective tissue. The inner surface is smooth and glistening with the pattern of fabric shining through. In cross sections the Marlex fibers appeared in columns of about 40 fibers regularly separated by septa that penetrated the width of the prosthesis. The encasing fibrous connective tissue on the inside equalled the width of the fabric while that on the outside was 3 times as wide as the fabric.

In Fig. 3 is shown a Marlex prothesis in place for 12 months. A segment of the aorta is continuous with the bypass prosthesis 10 cm long that is anastomosed to the distal abdominal aorta. The prosthesis is encased in a film of tissue with the structure of the fabric shining through (a). In microscopic preparations the pattern was somewhat different in sections cut longitudinally

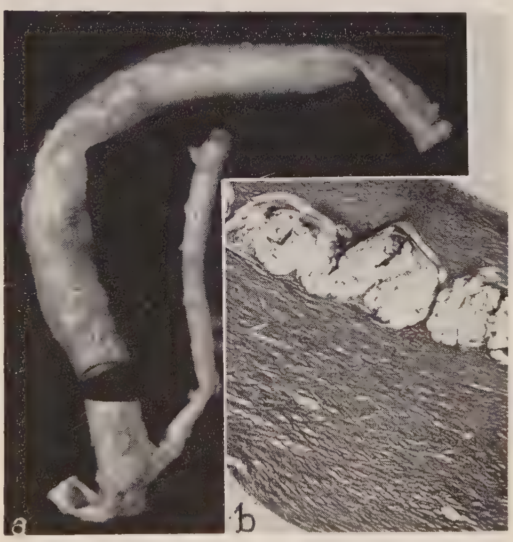

FIG. 2. Marlex prosthesis in place for 9 mo. Prosthesis is encased in fibrous connective tissue. Inner surface is smooth and glistening with pattern of the fabric shining through (a). In microscopic preparations, on cross section, the Marlex fibers appear to be in columns of about 40 fibers regularly separated by septa that penetrate the width of the prosthesis. The encasing fibrous connective tissue on the outside is about 3 times the width of the fabric; that on the inner surface equals width of fabric (b). (Weigert's elastic tissue stain × 50.)

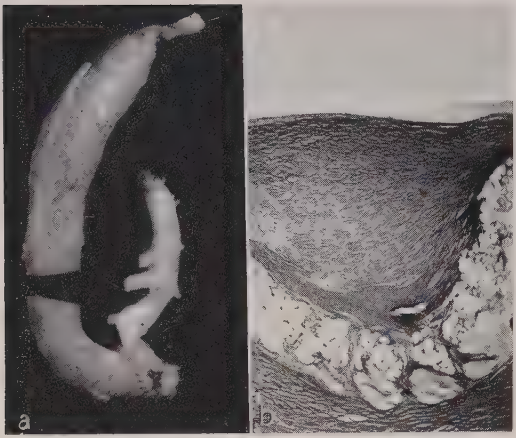

FIG. 3. Marlex prosthesis in place for 12 mo. Prosthesis is encased in a film of tissue through which structure of the fabric shines through (a). In a microscopic preparation cut longitudinally (b), the encasing fibrous connective tissue dips between the crests of the weave. At bottom of wedge between the crests there is osteoid and osseous tissue. (Weigert's elastic tissue stain × 50.)

and transversely. When cut longitudinally, the anisotropic, round or oval Marlex fibers, 6 to 10 rows deep, formed a wavy pattern with convex and concave areas alternating. The weave was of even width with the fibers close and with almost no space between them. A pink coagulum or ground substance and some elongated cell nuclei appeared here and there between the fibers. At intervals, complete or incomplete septa containing blood vessels or endothelium lined spaces extended between the outer and inner surfaces of the prosthesis. Both the outer and inner surfaces of the fabric were covered by a band of hyalinizing fibrous connective tissue composed of coarse collagenous bundles containing few elongated cell nuclei and no elastic fibers. This encasing fibrous connective tissue dipped between the crests of the weave filling the space and was thinner over the crests. At the bottom of the wedge between crests of the weave osteoid or osseous tissue formation was noted (b). The plaques of osteoid were more numerous on the intimal surface of the prosthesis than on the adventitial surface. The width of the encasing connective tissue was about that of the prosthesis except at the crests on either surface where it was thinner. In places a coagulum of varying widths was attached to the intimal surfaces and contained some cell nuclei.

Comment. For evaluation of a vascular prosthesis the experiment should be designed to simulate the planned clinical use of the graft. The method of bypass in this experiment seemed acceptable since the size of the vessel and length of the graft closely approximate those used in femoral-popliteal bypass in the human. Experimentally the prosthesis has proved suitable; however, there are adverse properties of the material which preclude its clinical use. The physical properties of the multi-filament yarn resisted close knit. Also the usability of the prosthesis is somewhat limited because the low melting point of the plastic prevents autoclaving. The physical properties of this material also present technical problems of handling at operation. Because this material lacks resiliency, the crimp in the prosthesis and thereby the protection against kinking is lost with stretching. Furthermore, the cohesiveness of the material impedes anastomotic suturing.

Summary. A vascular prosthesis of Marlex was implanted as a long aortic bypass graft in dogs and left in place up to 12 months. After 3 months of implantation, the inner surface of the prosthesis had a smooth lining and was firmly attached through the interstices to the periprosthetic tissue. None of the implanted prostheses exhibited stenosis, aneurysm or plaque formation. The inner and outer surfaces of the fabric were covered by a band of hyalinizing fibrous connective tissue. Plaques of osteoid were numerous on the intimal surface. Although the Marlex prosthesis functions adequately as a vascular substitute, unsatisfactory physical properties of this material limit its clinical use.

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1. Halpert, B., De Bakey, M. E., Jordan, G. L., Jr., Henley, W. S., *Surg. Gynec. Obst.*, v111, 659.

2. Usher, F. C., Ochsner, J. L., *Surgery,* 1959, v46, 718.

3. McCune, W. S., Blades, B., *Ann. Surg.*, 1951, v134, 769.

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Ecology of Rocky Mountain Spotted Fever. I. *Rickettsia rickettsii* Recovered from a Cottontail Rabbit from Virginia.*† (26581)

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Certain species of terrestrial mammals are known to be essential hosts for perpetuation of the ixodid ticks which are vectors of spotted fever rickettsiae. Strong presumptive evidence indicates that these mammals are probably important hosts of *Rickettsia rickettsii*, but actual recovery of the organisms from a native species of wild mammal has been confirmed only once in North America(1). Between May and December 1960, 10 wild cottontail rabbits, *Sylvilagus floridanus*, were trapped in various regions in Virginia and sent alive to the Walter Reed Army Inst. of Research. The present paper reports the recovery and characterization of a strain of *R. rickettsii* from one of these animals.

Methods. Each animal was bled for serum, spleen and liver were removed aseptically, and one-half of each tissue was used to prepare a 20% suspension in Snyder I diluent(2) containing 100 units penicillin and 20 μg streptomycin per ml. The remaining portions of whole tissue were stored at -70°C. Aliquots of the suspension were used for inoculation of guinea pigs, embryonated hens' eggs and blood agar slants. The guinea pigs were observed daily for 14 days for fever and for scrotal reaction. When guinea pigs developed fever (104°F or higher) for 2 consecutive days, a suspension of their blood, spleen and liver was passed to 3 guinea pigs. If the guinea pigs showed no fever or scrotal reaction by the 10th day, one was sacrificed, and its tissues passed to 3 guinea pigs. All surviving guinea pigs were bled 21-28 days after inoculation in order to test their sera for presence of complement-fixing antibodies employing spotted fever group-reactive antigens and, in some instances, specific washed rickettsial antigens (3).

The eggs were candled daily for 12-14 days, and the yolk sac of each embryo found dead after the second day was harvested aseptically, smeared and stained by Macchiavello's method. If rickettsia-like bodies were noted, the yolk sac was passed to eggs. The yolk sac of any dead embryo which had no sign of infection, *i.e.*, no visible rickettsia-like organisms, was passed to more eggs because of the possibility that the number of organisms was so meager that they could not be found by routine microscopic examination. If none of the embryos died by the 10th-12th day, a "blind" passage of one of the yolk sacs was made to other eggs. An isolation attempt in eggs and guinea pigs was not considered negative until 3 serial passages had been performed.

Guinea pigs which had recovered from a suspected rickettsial infection were challenged with the Bates strain of *R. rickettsii*. (The Bates strain was recovered in 1956 in this laboratory from a human case of Rocky Mountain spotted fever.)

Results. Data on attempts to recover *R. rickettsii* from cottontail rabbits trapped in Virginia are summarized in Table I. A suspension prepared from the spleen and liver of Rabbit 1, inoculated into the yolk sacs of embryonated eggs, caused death of 3 of 6 embryos on the 8th day. Organisms resembling rickettsiae were seen in smears of the yolk sacs, and this material served to establish the strain in serial egg passage. One of the 2 guinea pigs originally inoculated with the rabbit material had fever on the 6th day. A sus-

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† The materials for this study were collected in the field by J. T. Banks and T. M. Mullmann. The following individuals were instrumental in securing material and data: R. E. Dorer, principal investigator, J. T. Lamb, Jr., co-investigator, R. R. Gerhardt, F. N. Swink, Jr., G. Blank, and P. Woronecki.

TABLE I. History and Laboratory Studies of Cottontail Rabbits *(Sylvilagus floridanus)* from Virginia (1960).

Rabbit No.	Locality trapped	Date trapped	State of maturity	Serum titer*	Recovery of *R. rickettsii*
1	Farmville	6 May	Juvenile	<5	+
2	Leesburg	23 June	"	320	0
3	Charlottesville	3 July	"	<5	0
4	"	10 July	"	<5	0
5	"	16 July	"	<5	0
6	Camp Pickett	18 Aug.	"	20	0
7	Covington	18 Aug.	Adult	40	0
8	Camp Pickett	25 Aug.	Juvenile	<5	0
9	" "	13 Oct.	Adult	10	0
10	Maurertown	9 Dec.	"	<5	0

* Reciprocal of dilution of serum reacting with 4 units of group-reactive spotted fever antigen to fix complement.

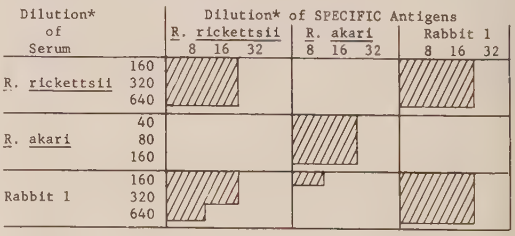
pension of blood, spleen and liver from this animal, sacrificed on the 2nd day of fever, served to establish the agent in serial guinea pig passages.

Behavior in guinea pigs: Following inoculation with established passage material, guinea pigs developed fever (104°F-105°F) which commenced on the 5th or 6th day, and persisted through the 10th or 11th day. None of the animals died, and only occasionally did any of them exhibit a grossly detectable tunica reaction manifested by definitely demonstrable adhesions.

Behavior in embryonated eggs: Standardized suspensions prepared from infected yolk sacs contained $10^{6.5}$ egg LD_{50}s when 0.2 ml of serial 10-fold dilutions were titrated in 7-day-old embryonated eggs. The lower dilutions caused death of the embryos in 4-5 days. Stained smears prepared from the yolk sacs usually contained 1-10 organisms/oil immersion field. Such infected yolk sac suspensions had infectious titers of about 10^{-7} when titrated in guinea pigs; animals which had 2-3 consecutive days of fever were considered to be infected since their convalescent sera invariably fixed complement in the presence of spotted fever group antigen.

Serologic behavior: Sera from guinea pigs which had been infected with the Rabbit 1 agent fixed complement in the presence of 2-4 units of group-reactive antigen prepared from yolk sacs infected with the Bitterroot strain of *R. rickettsii* or the 246 strain of *R. sibericus*. The Rabbit 1 guinea pig antiserum failed to react with 4 units of epidemic-murine typhus group antigen, scrub typhus, or Q fever antigen.

Group-reactive antigens prepared from yolk sacs infected with the Rabbit 1 agent fixed complement in the presence of convalescent sera from guinea pigs infected with the Rabbit strain, as well as *R. rickettsii* (2 strains), *R. akari*, *R. conorii* (3 strains), *R. australis*, *R. sibericus*, and the agents of maculatum disease and Indian tick typhus. Rabbit 1 antigens did not fix complement in the presence of sera from animals immune to *R. prowazeki*, *R. mooseri*, *R. tsutsugamushi* or *R. burneti*. Thus, the behavior of the new agent in complement fixation tests identified it as a member of the spotted fever group of rickettsiae. Moreover, specific washed rickettsial antigens prepared from yolk sac-propagated *R. rickettsii*, *R. akari*, and the Rabbit 1 agent distinguished the latter from *R. akari*, and identified it as a strain of *R. rickettsii* (Fig. 1). None of these specific antigens reacted with convalescent sera from guinea pigs infected with *R. sibericus*, *R. conorii*, *R. australis* and maculatum disease.



* Reciprocal of dilution.

FIG. 1. Results of reciprocal cross complement fixation tests employing specific washed antigens.

TABLE II. Results of Cross-immunity Tests in Guinea Pigs: Rabbit 1 *vs R. rickettsii, R. akari* and Maculatum Agent.

	Guinea pigs immune† when challenged with:			
Immunizing strain*	Rabbit 1	*R. rickettsii*	*R. akari*	Maculatum
Rabbit 1	6/6	6/6	6/6	6/6
R. rickettsii	6/6	6/6	—‡	—
R. akari	0/6	—	6/6	—
Maculatum agent	0/6	—	—	6/6
None (control)	0/4	0/4	0/4	0/4

* Standard strains and sources: *R. rickettsii*—Bates, a human case; *R. akari*—MK, from human case, New York; Maculatum agent—from tick, *Amblyomma maculatum.*

† Immune: No fever or scrotal reaction on challenge.

‡ Not done.

Cross-immunity: Table II summarizes a guinea pig cross-immunity test using the Rabbit strain and those agents of the spotted fever group known to occur naturally in North America. Initial infection with the Rabbit 1 agent protected all of the animals against a challenge with 100 guinea pig ID_{50}s of *R. rickettsii, R. akari,* maculatum disease and the homologous agent. Furthermore, those animals infected with *R. rickettsii* resisted challenge with the Rabbit 1 organism. On the other hand, an initial infection with *R. akari* or maculatum disease did not protect against a Rabbit 1 challenge. Thus, there was a one-way cross-immunity, *i.e.,* Rabbit 1 infection protected against challenge with maculatum disease or *R. akari,* but these agents did not immunize guinea pigs to infection with the Rabbit strain.

Behavior in mice: Intra-abdominal inoculation of albino mice with 10% Rabbit 1-infected yolk sac suspension failed to cause any apparent disease. The mice, however, were protected against challenge with 100 lethal infectious doses of the Hartford strain of *R. akari.*

Intravenous injection of infected yolk sac suspensions in concentrations of 5% or greater caused death of 10-12 g mice in 24 hours. Thus, the Rabbit strain has a toxic effect for mice similar to that reported by Bell and coworkers for their 3 strains of spotted fever rickettsiae(4).

Re-isolation: Although there was no doubt as to the validity of rabbit tissues as the source of the Rabbit 1 strain, the original stored tissues were thawed and tested, and a second line of the agent was recovered in eggs and guinea pigs inoculated with this material.

Discussion. The recovery of a strain of rickettsiae from a specimen of a species of wild mammal genus, long suspected to be a natural host for *R. rickettsii* in North America, not only warrants the fullest possible characterization of its biological properties, but also provides concrete evidence for the potential role of cottontail rabbits as hosts of *R. rickettsii* and possible donors of these organisms to ticks.

This strain of rickettsia is classified as *R. rickettsii* on the basis of the following observations. Its morphology is entirely consistent with that of rickettsiae. It grows intracellularly and fails to multiply within media containing no living cells. It produces a transmissible infection in guinea pigs, but fails to produce overt infection in adult albino mice, although the latter may succumb to the toxic effect of sufficiently large doses of viable organisms. Its behavior in cross-immunity tests in guinea pigs distinguishes it from known North American agents of the spotted fever group, other than *R. rickettsii.* Its serologic behavior in complement fixation provides the crucial evidence which (a) classifies it as a member of the spotted fever group and (b) identifies it as *R. rickettsii* when specific washed rickettsial antigens are prepared from it and tested against appropriate antisera. Likewise, infection with this agent produces in guinea pigs antibodies whose behavior in complement fixation identifies the agent as *R. rickettsii.* Although reciprocal cross-neutralization of toxicity for mice might provide further evidence of the identity of this organism, the observations cited above are considered to be sufficient.

With respect to the epidemiology of spotted fever, it is of interest to note that the behavior of this strain in guinea pigs is not unlike that of some strains of *R. rickettsii* recovered from human cases of the disease. The implications are obvious. With reference to cottontail rabbits, Jellison(5) noted the highly significant degree of correlation between the geographic distribution of species of *Sylvilagus* and the occurrence of human cases of spotted fever in the United States. Furthermore, *Sylvilagus* spp. are frequently hosts for the immature stages of *Dermacentor variabilis* and *D. andersoni*, which, as adults, may transmit spotted fever to man.

In reporting their recovery of *R. rickettsii* from a naturally infected meadow mouse, *Microtus pennsylvanicus*, taken in Virginia, Gould and Miesse(1) discussed the possible significance of their finding and reviewed pertinent prior studies of previous workers in the United States and Latin America.

In the course of the present study, rickettsial strains have also been recovered from 1 of 330 *Peromyscus leucopus* and 1 of 2 *Pitymys pinetorum*. Although their behavior in complement fixation classifies these strains to be members of the spotted fever group, neither has yet been sufficiently characterized to permit precise species identification.

Serologic studies revealed the presence of complement-fixing antibodies for the spotted fever group in 4 of the 10 wild rabbits (Table I). In addition, a limited survey of the sera of certain other wild mammals revealed similarly reacting antibodies in the sera of 1 or more specimens of red fox, gray fox, opossum, raccoon, white-footed wood mouse, and deer. These findings are interpreted tentatively as evidence of past spotted fever infection.

Further studies are necessary in order to evaluate more precisely the role of other mammals as well as rabbits in the ecology of *R. rickettsii*. Current field and laboratory studies are thus aimed at answering such questions as the following: What is the most sensitive method for detecting present or past infection with *R. rickettsii* in a particular species of mammal? Can one recover viable *R. rickettsii* from mammals whose serum contains complement-fixing antibody? For how long a period of time can an individual mammal serve as a donor of *R. rickettsii* to noninfected ticks that feed upon it? Is there a relationship between age of a rabbit and time that it usually becomes infected?

Summary. From a naturally infected wild cottontail rabbit, *S. floridanus*, trapped in Virginia, an organism identified as *R. rickettsii* has been recovered and characterized. The role of cottontail rabbits and certain other native wild mammals in the ecology of Rocky Mountain spotted fever is discussed briefly in the light of this and other related findings.

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1. Gould, D. J., Miesse, M. L., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 558.
2. Jackson, E. B., Smadel, J. E., *Am. J. Hyg.*, 1951, v53, 326.
3. Smadel, J. E., *Diagnostic Procedures for Virus and Rickettsial Diseases*, Am. Public Health Assn., 2nd Ed., 1956, 513.
4. Bell, E. J., Stoenner, H. G., *J. Immunol.*, 1960, v84, 171.
5. Jellison, W. L., *Public Health Rep.*, 1945, v60, 958.

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Serological Comparisons Between Infectious Canine Hepatitis Virus and Human Adenovirus Types. (26582)

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The adenovirus group is a large family of infectious agents which possesses certain common biological properties, including a soluble group-reactive complement-fixing antigen(1,2). Recent studies have disclosed biological similarities between infectious canine hepatitis virus (ICHV) and certain members of the human adenovirus group(3, 4,5). Also, adenovirus types 2, 3, 4 and 7 have been reported to produce an asymptomatic infection in experimentally exposed dogs(6). Additional studies were made and this paper presents further evidence of the antigenic relationships between ICHV and certain human adenoviruses as shown by complement-fixation, indirect hemagglutination, virus neutralization, and gel-diffusion precipitin methods.

Methods. Paired canine sera were obtained from purebred beagles that had been given a single inoculation of adenovirus type 4 or Cornell strain 1 ICHV subcutaneously. Sera were taken at time of inoculation and again approximately 30 days afterwards. Other antisera for adenovirus were: (1) human type 2 antiserum supplied by Dr. W. P. Rowe (Nat. Inst. Health, Bethesda, Md.) (2), acute and convalescent human adenovirus type 4 antiserum obtained from Dr. R. R. Gutekunst (Great Lakes Naval Biological Research Unit, Great Lakes, Ill.), and (3) guinea pig adenovirus antiserum prepared in this laboratory by inoculating 0.2 ml of an infective tissue culture suspension of type 4 intranasally, followed 2 weeks later by an inoculum of 1 ml intraperitoneally. The guinea pigs were bled for serum 21 days after final inoculation.

Complement-fixation (CF) tests. ICHV antigens for CF tests were prepared from 7-day cultures of dog kidney cells infected with Cornell strain 1 ICHV. Cultures were frozen and thawed 6 times, clarified by centrifugation at 2000 rpm for 10 minutes and pooled in 2 ml portions which were then stored at –20°C until used. Adenovirus type 4 CF antigen was prepared from infected HeLa cell cultures. After complete cell degeneration had occurred, cultures were frozen and thawed, clarified by light centrifugation, pooled, then stored at –20°C in 5 ml portions.

In all tests, paired sera were tested simultaneously. Serial 2-fold dilutions of heat-inactivated serum in 0.25 ml amounts were incubated for 2 hours at 36°C with 4 units of either ICHV or adenovirus antigen in 0.25 ml and 2 full units of complement contained in 0.5 ml of Kolmer saline. A unit of antigen had been previously determined by titration. After 2 hours incubation at 36°C, 0.5 ml of a prewarmed mixture of 2 units of hemolytic amboceptor and 2% sheep erythrocytes was added. The test was read after further incubation for 30 minutes and endpoints were then recorded as the highest serum dilution showing at least 50% fixation by visual observation. Appropriate controls were included.

Indirect hemagglutination tests. Hemagglutination procedures have been previously reported(7). Serial 5-fold serum dilutions were heat-inactivated and mixed in equal volumes of 0.2 ml with 1% tanned sheep erythrocytes sensitized with ICHV or adenovirus type 4 antigens. After 2 hours incubation at room temperature (approximately 26°C) the endpoints were recorded as the highest serum dilution giving a 2-plus hemagglutination pattern.

Virus neutralization tests. For ICHV neutralization tests, serial 5-fold dilutions of heat-inactivated serum were mixed with an equal volume of virus suspension containing 100 to 300 $TCID_{50}$ per 0.1 ml. After incubation for 1 hour at 36°C, virus-serum mixtures were inoculated into tubes of dog kidney cells using an inoculum of 0.2 ml per tube and 4 tubes for each dilution. Assay for adenovirus antibody in certain canine sera was kindly performed by Dr. W. P. Rowe. Inoculated cell cultures were maintained at 35°C in stationary racks. Tests were finally read when at least 50% degeneration was evident in the virus control cultures showing 100 to 300 $TCID_{50}$.

Agar-gel precipitin tests. Ouchterlony gel-precipitin tests were carried out according to a modification of the procedure described by Brown and Crick for antigenic analysis of foot-and-mouth disease virus(8). Antigen was prepared from 7-day cultures of infected cells which had been frozen and thawed 6 times and then centrifuged at 2000 rpm for 10 minutes. Clearest precipitin bands were obtained with virus preparations which had been concentrated 10 times by dialysis against 20% polyvinylpyrrolidone (Armour) at 4°C. Noble agar (Difco) at a concentration of 1.5% in buffered saline (pH 7.4), con-

TABLE I. Serological Comparisons of Infectious Canine Hepatitis and Human Adenoviruses.

		Serum antibody titers (reciprocal of dilution)					
		Complement-fixation		Hemagglutination		Neutralization	
Paired sera*		Adeno.†	ICHV	Adeno.	ICHV	Adeno.	ICHV
Human adeno. 4	(A)	4	4	125	15,625	<5	30
	(C)	256	128	625	78,125	>625	30
Human adeno. 2	(A)	<4	<4	<5	<5	NT	<5
	(C)	128	128	125	15,625	"	<5
Guinea pig adeno. 4	(A)	<4	<4	<5	<5	<5	<5
	(C)	128	32	125	78,125	>25	<5
Canine adeno. 4	(A)	<4	<4	<5	<5	<4	<5
	(C)	64	16	25	625	>4	<5
Idem	(A)	<4	<4	NT	NT	<4	<5
	(C)	32	4	"	"	>4	<5
"	(A)	<4	<4	"	"	4	<5
	(C)	32	8	"	"	>4	<5
"	(A)	<4	<4	"	"	4	<5
	(C)	8	4	"	"	>4	<5
"	(A)	<4	<4	"	"	<4	<5
	(C)	64	16	"	"	>4	<5
Canine ICHV	(A)	<4	<4	<5	<5	<5	<5
	(C)	<4	128	<5	390,625	<5	10,000
Idem	(A)	<4	<4	NT	NT	<5	<5
	(C)	<4	128	"	"	<5	300,000
"	(A)	<4	<4	<5	<5	<5	<5
	(C)	<4	16	<5	15,625	<5	25,000
"	(A)	<4	<4	NT	NT	<5	<5
	(C)	<4	64	"	"	<5	300,000
"	(A)	<4	<4	"	"	<5	<5
	(C)	<4	128	"	"	<5	700,000

* Serums taken during acute phase or preinoculation (A) and convalescent or postinoculation (C).

† Antigen used.

taining 0.01% thiomersalate (final concentration) and 1:300,000 methyl orange, was poured in 15 ml portions into 9 cm Petri plates and held at 4°C. Plates were placed at room temperature for 8 to 12 hours before use. Cups 7 mm in diameter and 7 mm apart were cut from the agar with the aid of a cutting template. These held approximately 0.05 ml and were filled 2 or 3 times within a 2-hour period. After filling, plates were kept at 35°C in a humidified chamber and precipitin bands were usually evident within 12 to 24 hours, however, the test was finally read after 72 hours when clearest lines were observed.

Results. Results of serological comparisons of Cornell strain 1 ICHV with human adenovirus types 2 and 4 are shown in Table I. Human adenovirus serum CF titers were nearly the same when ICHV antigen was used in place of the homologous antigen in the test. Adenovirus antisera produced in dogs and guinea pigs, however, exhibited lower titers to the heterologous (ICHV) antigen. In contrast, adenovirus CF antigens failed to fix complement with immune canine hepatitis sera, indicating type-specificity for ICHV antibody.

Antigens participating in the indirect hemagglutination test, which are virtually the same as those responsible for complement-fixation(9), gave results similar to those obtained in the CF test, for adenovirus immune sera agglutinated erythrocytes sensitized with both adenovirus and ICHV antigens, while ICHV immune sera reacted only with the homologous test antigen. Higher adenovirus antibody titers were observed when ICHV-

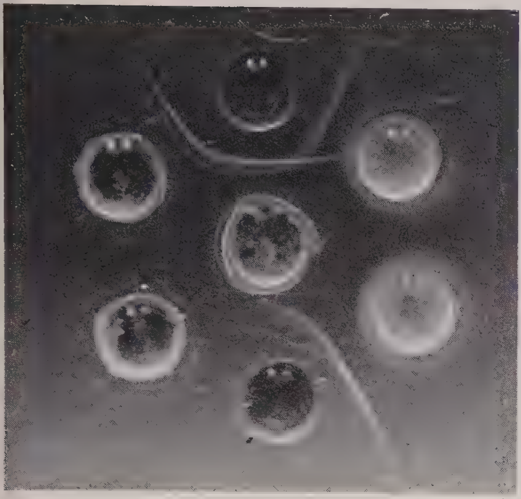

FIG. 1. Gel precipitin test showing unilateral serological relationship between ICHV and adenovirus type 4. ICHV (top well), adenovirus type 4 (bottom well), immune ICH serum (center and left wells), immune adenovirus type 4 serum, human origin (right wells).

sensitized cells were employed. These probably resulted from larger amounts of cell-adsorbed antigen since serum titers are related to the amount of antigen adsorbed onto erythrocytes, provided that all receptors are not saturated(5). The CF antigen titer of the adenovirus preparation used for sorption was 8-times less than the ICHV titer.

ICHV and adenovirus types 2 and 4 were specifically neutralized only by homologous antisera. Dogs inoculated with adenovirus type 4 developed significant neutralizing antibodies only against the homologous virus, for sera from such dogs failed to neutralize ICHV. An interesting finding was the presence of adenovirus antibodies in some dogs, preinoculation sera.

The unilateral serologic relationship between ICHV and adenoviruses was readily demonstrated by employing Ouchterlony precipitin tests. Fig. 1 shows a typical result when immune adenovirus type 4 serum and ICHV-immune serum were tested against adenovirus type 4 and ICHV. At least 3 precipitin lines were formed between adenovirus type 4 immune serum and ICHV, while none appeared between ICHV immune serum and the adenovirus. Insufficient resolution was achieved to determine the precise number of cross-reacting antigens; however, adenovirus type 2 antiserum formed at least 3 and probably 4 bands with ICHV.

Discussion. Many of the basic criteria for placing ICHV taxonomically within the adenovirus group seem to have been adequately met. Since Kapsenberg's report of the serological relationship between ICHV and certain human adenoviruses(3), further studies have shown that the relationship is unilateral, for adenovirus antiserum reacts with ICHV in complement-fixation and in gel-diffusion precipitin tests but canine hepatitis antiserum does not react with adenovirus antigens(4). The serological findings reported here confirm the antigenic relationships and have further demonstrated the multiplicity of shared antigens between the 2 groups. In our experience, human sera containing adenovirus antibodies fixes complement with ICHV and adenovirus antigens to nearly the same degree. In contrast, canine sera containing adenovirus antibodies consistently showed higher complement-fixation titers when the homologous antigen was used, demonstrating a degree of type specificity. Similar findings have been reported for various human adenovirus sera produced in rabbits(1) and for a newly described mouse virus related to the adenovirus group(10).

Since viruses that share group antigens with the adenoviruses have been isolated from monkeys and chimpanzees(1,2), cattle(11), fowl(12), rodents(10), and now apparently the dog, biologic comparisons become all the more important, for the sharing of antigenic determinants does not in itself allow taxonomic grouping. The well-known Weil-Felix reaction depends upon antigens shared by such biologically distinct groups as proteus and rickettsiae. Also, unilateral antigenic relationships based upon immunologic studies have been demonstrated between certain staphylococci and *Listeria monocytogenes* (13). Additional evidence which supports the inclusion of ICHV into the adenovirus group is shown in Table II.

Occasional reports that man and monkeys may become either asymptomatically or manifestly infected with ICHV(17,18) have been based, primarily, upon retrospective

TABLE II. Biological Characteristics Shared by Human Adenoviruses and Infectious Canine Hepatitis Virus.

Characteristics		Adenovirus*	ICHV†
Soluble group-reacting CF antigen		+	+
Ether resistance		+	+
Cultural:	Cytopathogenicity—intranuclear inclusions, primarily DNA	+	+
	Acid production by infected cell	+	+
	Intranuclear ''crystals''—some strains	+	+
	Latent period 14 to 21 hr	+	+
	Greater cell-association virus	+	+
	Cell-detachment factor, soluble ''toxin''	+	
Stability:	Prolonged at 4°C, rapid inactivation 56°C	+	+
	pH 3 to pH 9 (30 min., room temperature)	+	+
Lack of pathogenicity for small laboratory animals		+	+
Size: 50-65 $m\mu$ (intracellular)		+	+
Hemagglutinin for erythrocytes for various animal species		+	+
Isolation from tonsils and urine of asymptomatic carriers		+	+

* (1,2) † (3,5,14,15,16)

methods which employed the complement-fixation test. In view of the antigenic relationships between ICHV and human adenoviruses, it would seem necessary to re-evaluate these findings, especially since increases in specific neutralizing antibody or virus isolations have not been reported.

Summary. Antigenic relationships between infectious canine hepatitis virus (ICHV) and certain human adenoviruses have been confirmed by complement-fixation, indirect hemagglutination and gel-diffusion precipitin tests. The serological relationship was unilateral, since antisera against human adenoviruses react in various tests with ICHV antigen, while specific antisera to ICHV failed to react with group-reactive human adenovirus antigens (types 2 and 4). There was no specific cross-neutralization between ICHV and the adenovirus types examined. Certain biologic properties shared by ICHV and the human adenoviruses have been compared, and these suggest that ICHV should be included taxonomically within the adenovirus group.

1. Heubner, R. J., Rowe, W. P., Chanock, R. M., *Ann. Rev. Microbiol.*, 1958, v12, 49.

2. Ward, T. G., *Prog. Med. Virol.*, 1959, v2, 203.

3. Kapsenberg, J. C., PROC. SOC. EXP. BIOL. AND MED., 1959, v101, 611.

4. Heller, L. A., Salenstedt, C. R., *Virology*, 1960, v11, 640.

5. Carmichael, L. E., Doctoral Thesis, Cornell, 1959.

6. Sinha, S. K., Fleming, L. W., Scholes, S., *J. Am. Vet. Med. Assn.*, 1960, v136, 481.

7. Carmichael, L. E., Sarkar, S., *Cornell Vet.*, 1958, v49, 504.

8. Brown, F., Crick, J., *Virology*, 1958, v5, 133.

9. Ross, E., Ginsberg, H., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 501.

10. Hartley, J. W., Rowe, W. P., *Virology*, 1960, v11, 645.

11. Klein, M., Earley, E., Zellat, J., PROC. SOC. EXP. BIOL. AND MED., 1959, v102, 1.

12. Burmester, B. R., Sharpless, G. R., Fontes, A. K., *J. Nat. Cancer Inst.*, 1960, v24, 1443.

13. Welsheimer, H. J., *J. Bact.*, 1960, v79, 456.

14. Fastier, L. B., *Nature*, 1957, v179, 420.

15. Hagan, W. A., Bruner, D. W., *The Infectious Diseases of Domestic Animals*, 4th ed., Comstock, Ithaca, N. Y., 1961.

16. Leader, R. W., Pomerat, C. M., Lefeber, C. G., *Virology*, 1960, v10, 268.

17. Martin, L. H., Delange, B., *Arch. Inst. Past. Maroc.*, 1956, v5, 56.

18. Schindler, R., Mohr, W., *Dtsch. med. Wschr.*, 1959, v84, 2080.

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Renal Clearance of Intravenously Administered Vitamin B_{12}. (26583)

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Previous studies have shown greater excretion of parenterally administered vit. B_{12} among young subjects than old. Furthermore, the decrement in vit. B_{12} excretion with age seemed to parallel agewise decrements observed in various renal functions(1). Since no direct experiments were performed to estimate whether rates of removal of vit. B_{12} from the plasma by the kidneys were reduced in older individuals, the following studies were carried out.

Materials and methods. Simultaneous clearances of B_{12} and inulin were measured in 2 groups of ambulatory subjects selected by clinical and laboratory tests as free from renal or hepatic disease as previously described(2). In the 6 subjects of Group A and the 10 of Group B, constant plasma levels of B_{12} and inulin were maintained following initial priming doses* by continuous infusion at a rate held constant by a Bowman pump. After 30 minutes of equilibration, 4 or more 15-minute clearance periods were run on each subject. All subjects were catheterized; their bladders were washed out with distilled water and air at the beginning and end of each period.

In Group A, the plasma was heated with buffer solution to liberate bound B_{12} from protein(3). Protein was removed by centrifugation and the supernatant assayed for B_{12} activity. This technic measured total B_{12}; *i.e.*, both free and that bound to plasma protein. In Group B, the plasma was dialyzed and the assay run on the dialysate, so that only free B_{12} was measured.

Triplicate microbiological assays using *Lactobacillus leichmannii 4797*(4) were averaged to estimate plasma and urine levels of B_{12}. Mendelsohn and Watkin(5), using an *L. leichmannii* assay, have reported recoveries of added B_{12} from sera averaging 98.6 ± 3.13%. Experiments were conducted to determine the reproducibility of the vit. B_{12} assay. The variability due to technical error can be conservatively estimated to be within ±20% of the true value. Although no information on the over-all accuracy of B_{12} clearances is available, it is entirely possible that the error of individual portions of the clearance determination may be additive leading to a total error as great as 20-30%.

Inulin was determined in plasma and urine by the method of Harrison(6). In this laboratory, Davies and Shock(7) reported standard deviations about the mean inulin clearance of 4.7 to 7.0% between successive 10 minute periods during the same test.

Results. Investigations of B_{12} clearances at endogenous plasma levels of B_{12} (0.2 to 0.3 mμg/ml) in 3 normal subjects revealed no measurable quantities of B_{12} in the urine. The minimum amount of B_{12} which could be determined by this method was 0.010 mμg. In the 6 subjects of Group A, total B_{12} plasma levels were increased to concentrations ranging from 0.3 to 2.4 mμg/ml by continuous intravenous infusion. Glomerular filtration rates ranged from 29 to 136 ml/min in these subjects.

At B_{12} plasma levels below 1 mμg/ml practically no B_{12} appeared in the glomerular filtrate or urine (Fig. 1). At levels above 1.5 mμg/ml large variations existed between plasma level and urinary B_{12}/ml of glomerular filtrate. However, at these low levels (0.4-2.4 mμg/ml) the error in estimating B_{12} is sufficiently large to warrant the inference that

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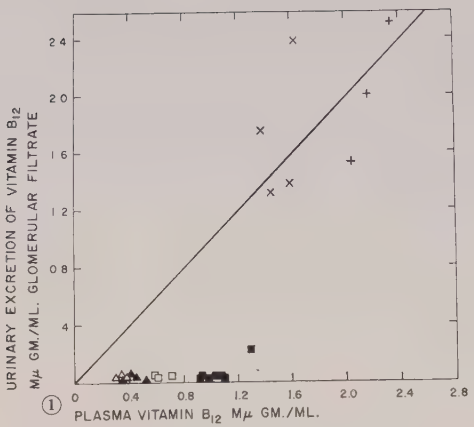
* The priming dose of inulin was calculated in the usual manner(2). The B_{12} primary dose was calculated for each subject as sufficient to provide the desired plasma level assuming that shortly after administration B_{12} was uniformly distributed in the extracellular fluid space as calculated from body weight.



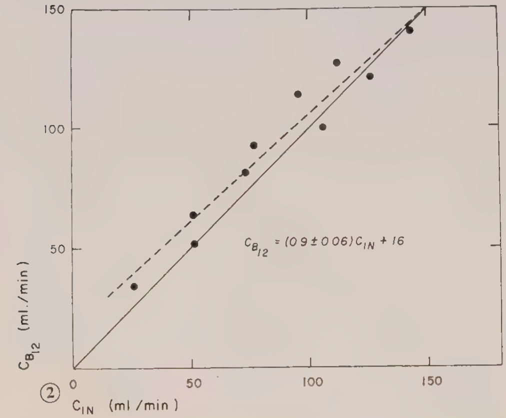


FIG. 1. Relationship between plasma level and urinary excretion of vit. B_{12} at low plasma levels. Individual clearance periods are plotted for 6 subjects as indicated by different symbols. At plasma levels less than 1.2 mμg/ml of B_{12}, excretion of B_{12} was very small and did not increase with plasma concentration.

FIG. 2. Relationship between inulin clearance and vit. B_{12} clearance in 10 subjects. Each point is the average of 4 clearance periods determined at constant plasma levels of B_{12} in each subject. Dotted line represents regression line calculated by the method of least squares. The regression equation is: Clearance of B_{12} (ml/min) = $(0.9 \pm 0.06)\ C_{In} + 16$. The experimental regression line (------) does not differ significantly from the regression (———) based on the theoretical identity of B_{12} and inulin clearances.

urinary clearance of B_{12} would be equivalent to inulin clearance if the free B_{12} content of the plasma could be accurately estimated.

In the 10 subjects of Group B, B_{12} plasma levels ranged from 12 to 26 mμg/ml. Glomerular filtration rates ranged from 25 to 160 ml/min (Table I). In Fig. 2, values obtained by averaging clearance data for B_{12} and inulin from 4 periods in each individual in Group B are presented. These average values indicate that B_{12} clearance is approximately that of inulin over a wide range of glomerular filtration rates at plasma levels ranging from 12 to 26 mμg/ml. Calculation of the regression of B_{12} clearance on inulin clearance based on data from 40 individual clearance periods in the 10 subjects yielded the following equation: Clearance of $B_{12} = (0.9 \pm 0.06)\ C_{In} + 16$. This is shown by the dashed line in Fig. 2. The slope of the regression does not differ significantly from 1.0.

Discussion. At low plasma levels (0.3 to 1.1 mμg B_{12}/ml), no B_{12} was detected in the urine. An explanation for this observation is that at low plasma levels vit. B_{12} is bound to plasma protein and therefore not filtered at the glomerulus. Indeed, *in vitro* experiments have indicated that at B_{12} levels of 0.5 to 1.0 mμg/ml, the B_{12} is bound to the alpha globulins(8-10). Furthermore, the low level of free B_{12} that may exist could not be detected by the assay method used. When concentration of vit. B_{12} was increased to 1 mμg/ml, the binding capacity of alpha globulin was exceeded and measurable amounts of the vitamin were found in the urine. The data obtained on the second group of subjects (Group B), in which vit. B_{12} level was raised to insure greater accuracy of determination and the plasma was dialyzed to separate the free from the bound B_{12}, showed that vit. B_{12} clearance was indistinguishable from glomerular filtration rate.

The impaired glomerular filtration rate observed in older subjects results in an elevated plasma level of vit. B_{12} for a longer period of time than that in younger individuals. Although the mechanism by which vit. B_{12} is presented to and retained by the tissues is unknown, this may provide a greater opportunity for the various tissues of the body to retain the vitamin in the older subjects than in the young.

Conclusions. 1. At plasma levels ranging from 0.3 to 1.5 mμg/ml urinary excretion of B_{12} is a function of degree of B_{12} binding by plasma protein. 2. At constant plasma levels between 12 and 26 mμg/ml of vit. B_{12}, urinary excretion of B_{12} in a given individual is proportional to plasma level and glomerular fil-

TABLE I. Simultaneous Inulin and B_{12} Clearances in 10 Subjects by Individual Periods at Plasma Levels of 12-26 mμg B_{12}/ml.

Subject	Age	Clearance, ml/min.	Period				Mean clearance
			I	II	III	IV	
Bn	17	Inulin	110.3	114.9	107.9	113.2	111.6
		B_{12}	122.5	121.4	133.8	131.6	127.3
Df	35	Inulin	131.1	134.4	122.3	114.7	125.6
		B_{12}	99.6	124.1	115.8	143.5	120.8
Cn	51	Inulin	160.5	140.7	128.7	141.5	142.8
		B_{12}	147.8	127.4	130.7	154.5	140.1
Ti	52	Inulin	51.9	49.7	56.1	47.2	51.2
		B_{12}	63.7	55.3	58.3	76.6	63.5
Le	53	Inulin	103.3	108.0	107.3	104.7	105.8
		B_{12}	86.4	115.0	83.0	117.3	100.4
Po	59	Inulin	73.4	76.1	68.5	76.1	73.5
		B_{12}	85.0	80.4	78.3	82.9	81.6
Ro	64	Inulin	99.5	98.5	94.7	89.5	95.6
		B_{12}	134.2	107.2	98.7	116.6	116.7
Sm	75	Inulin	50.8	50.8	51.5	53.1	51.6
		B_{12}	46.1	56.9	61.8	44.7	52.4
Fv	77	Inulin	66.6	84.1	78.3	80.1	77.3
		B_{12}	86.9	88.6	94.4	100.9	92.7
Mu	88	Inulin	25.8	26.8	25.5	25.9	26.0
		B_{12}	34.4	37.5	38.6	27.4	34.5

tration rate. 3. No relation of renal clearance of B_{12} with advancing age was noted except for that resulting from the gradual reduction in glomerular filtration rate with age.

1. Watkin, D. M., Long, C. A., Chow, B. F., Shock, N. W., *J. Nutrit.*, 1953, v50, 341.

2. Davies, D. F., Shock, N. W., *J. Clin. Invest.*, 1950, v29, 496.

3. U. S. Pharmacopaeia, 14th revision, 4th supplement, 1954, 10.

4. Skeggs, H. R., Nikkle, H. M., Valentik, K. A., Huff, J. W., Wright, L. D., *J. Biol. Chem.*, 1950, v184, 211.

5. Mendelsohn, R. S., Watkin, D. M., *J. Lab. and Clin. Med.*, 1958, v51, 860.

6. Harrison, H. E., PROC. SOC. EXP. BIOL. AND MED., 1952, v49, 111.

7. Davies, D. F., Shock, N. W., *J. Clin. Invest.*, 1950, v29, 491.

8. Mendelsohn, R. S., Watkin, D. M., Horbett, A. P., Fahey, J. L., *Blood,* 1958, v13, 740.

9. Pitney, W. R., Beard, M. F., Van Loon, E. J., *J. Biol. Chem.*, 1954, v207, 143.

10. Weinstein, I. B., Weissman, S. M., Watkin, D. M., *J. Clin. Invest.*, 1959, v38, 1904.

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Partial Purification of Erythropoietin by Alcoholic Fraction. (26584)

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A number of methods for concentration of the erythrocyte stimulating factor or erythropoietin in the plasma of anemic rabbits have been devised. Chemical methods of varying complexities have been employed and assays have been performed on both normal and abnormal animals(1). The method to be described utilized simple fractionation procedures and all assays were performed on normal adult albino rats. A preliminary report was made in 1958(2).

Methods. Production of anemia. Adult

male New Zealand rabbits 2-3 Kg were injected s.c. daily with 0.5 to 1.0 ml of a 2.5% solution of phenylhydrazine. Since the most active plasmas were obtained from animals with hematocrit values of 10-12% from 5-7 days after start of injections, it was necessary to measure the hematocrit values of the rabbits daily to obtain maximal erythropoietic titers. Blood was drawn by cardiac puncture, heparinized, centrifuged and the plasma frozen until used. Apparently heparin did not interfere, since similar activity was found in serum.

Normal male albino rats (Carworth Farms) weighing 180 to 200 g were used in all assays. Hematocrit values of tail blood were measured using Van Allen tubes and reticulocyte percentages were estimated by counting 1000 cells after staining with New Methylene Blue.

Approximately 0.3 mc of Fe^{59} (as FeCl$_3$ in 0.3 ml) was injected into the saphenous vein of the rat 24 hours after the plasma fraction or saline as control had been given by i.p. injection. Eighteen hours after Fe^{59} injection, 0.1 ml of blood was obtained from the tail, cells were separated, washed in saline and counted in a scintillation counter. Iron uptake values were expressed as per cent of total iron administered. In all Fe^{59} uptake studies, at least 4 rats were used in each group.

Alcohol fractionation. Plasma obtained from anemic rabbits was adjusted to pH 5.5, boiled for 15 minutes and filtered as described by Borsook(3). The opalescent filtrate was adjusted to pH 7.3 and ethanol added to 60% by volume. The alcohol was added slowly with stirring and temperature was maintained at 25°C. After standing 60 minutes, the white flocculent precipitate was easily separated by centrifugation and discarded. Ethanol was added to the supernatant to 80% concentration. The resultant 60-80% ethanol precipitate was separated by centrifugation after 30 minutes and was quite active. It was further purified by dissolving in water, adjusting to pH 5.5 and adding ethanol to 50% concentration. The precipitate was removed by centrifugation and discarded. Ethanol was added to the supernatant to achieve a concentration of 80%. This active 50-80% precipitate was separated by centrifugation after 30 minutes standing. By narrowing the alcohol range to 70 to 75%, for example, it was possible to achieve a greater degree of purity although a considerable loss of substance was experienced. For injection, all fractions were dissolved or suspended in saline up to a volume equivalent to that of the original plasma filtrate and injection volumes were maintained constant.

Various organs obtained from anemic rabbits were prepared for assay by grinding with an equal volume of saline, adjusting to pH 5.5, boiling for 15 minutes and filtering. The filtrate was neutralized and injected into rats as were the plasma fractions.

Results. Rats were injected s.c. 6 days a week for 4 weeks with 1 ml of the plasma filtrate obtained from anemic rabbits, 0-60% ethanol precipitate, 60-80% ethanol precipitate or saline (Fig. 1). In the groups receiving the plasma filtrate and the 60-80% precipitate significant increases in both reticulocytes and hematocrit values occurred. No significant changes appeared in the groups receiving either saline or 0-60% ethanol precipitate. When rats were injected s.c. 6 days a week with the untreated plasma from anemic rabbits, no increase in hematocrit was noted and death occurred in a few weeks. The reaction is probably immunologic and has been noted by Borsook(3) and Gordon (4).

A single i.p. injection of 4 ml of various active plasma fractions 24 hours before Fe^{59}

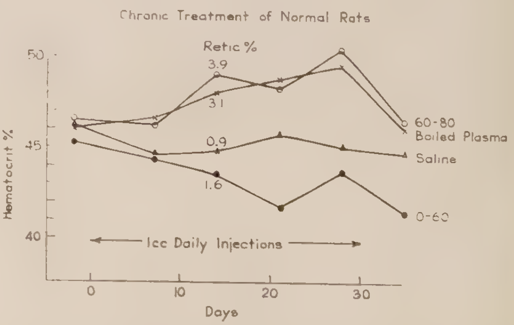

FIG. 1. Effects of boiled plasma filtrate, 60-80% ethanol precipitate, 0-60% ethanol precipitate and saline on normal rats.

TABLE I.

Fractions of anemic rabbit plasma	% Fe^{59} uptake	Amt of solids in fraction inj. (mg)
Control (saline)	33	—
Raw plasma	42	280.0
Boiled plasma filtrate	46	58.0
60-80% ethanol, pH 7.3	46	1.76
50-80% ” , pH 5.5	47	.16
70-75% ” , pH 7.3	44	.08

injection produced a 30-40% increase in Fe^{59} uptake by circulating red blood cells (Table I). Degree of concentration achieved by these relatively simple fractionation procedures is indicated by the reduction in solids of the various fractions (Table I). Greater purification could be achieved by narrowing the alcohol fractions, but results were not easily reproducible.

Table II lists 30 tissues, organs or body fluids from anemic rabbits which were tested for erythropoietic stimulating activity. Consistent, positive results were not obtained from any of these sources even though the plasmas obtained from the same animals were active. This finding confirms the work of Gordon *et al.*(5).

Despite the crude nature of all fractions, certain properties of the stimulating material could be established. It is water soluble, resists boiling for 15 minutes and is insoluble in 100% alcohol or acetone. It will not dialyze and can not be ultrafiltered across an alundum candle coated with 10% parlodion. It is active when administered by the usual parenteral route but inactive by mouth.

TABLE II. Sources Tested for Erythropoietin Activity.

Bone marrow
Spleen
Brain
Kidney
Liver
Skeletal muscle
Stomach
Intestine
Lung
Pancreas
Skin
Bone
Erythrocytes
Leucocytes
Embryo
Thymus
Lymph gland
Salivary ”
Mammary ”
Pituitary
Thyroid
Adrenal
Testis
Ovary
Urine
Bile
Lymph
Aqueous humor
Milk
Feces

Discussion. Ethanol fractionation of the boiled filtrate provided a simple and reproducible means of partial purification of the erythropoietic stimulating principle. Concentration of activity on a weight basis amounted to 3000 fold. An indication of the lack of purity is obtained by comparison of Tiselius electrophoretic migration patterns of the 50-80% ethanol precipitates obtained from normal rabbits and those obtained from anemic animals. Although the material from normal rabbits was inactive, the electrophoretic patterns were indistinguishable.

The use of chromatography columns composed of hydroxyapetite, celite or DEAE cellulose as a means of fractionation was inefficient in our hands. The procedures were elaborate and although some purification was achieved, a large amount of the active material was lost.

Summary. The erythropoietic stimulating principle present in the filtrate of plasma obtained from anemic rabbits was partially purified by ethanol fractionation. This material was active in increasing Fe^{59} intake of *normal* rats and in producing a reticulocytosis and hematocrit elevation in repeatedly injected *normal* rats. Fe^{59} uptake was stimulated by as little as 0.08 mg of the fraction representing a concentration of 3,000 fold over that occurring in the plasma.

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1. Gordon, A. S., *Physiol. Rev.*, 1959, v39, 1.

2. Grant, W. C., Linkenheimer, W. H., Berger, H., *Fed. Proc.*, 1958, v17, 59.

3. Borsook, H., Graybiel, A., Keighley, G., Windsor, E., *Blood* 1959, v9, 734.

4. Gordon, A. S., Piliero, S. J., Kleinberg, W., Freedman, H. H., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 255.

5. Gordon, A. S., Piliero, S. J., Tannenbaum, M., *Am. J. Physiol.*, 1955, v181, 585.

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Effect of Centrifugation at 20,000 × g on Lipid Distribution of Human Sera. (26585)

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Separation of very low-density lipid particles (including the chylomicra) from whole serum by high-speed centrifugation has led to interesting results from several laboratories. Bragdon(1) has shown that hypercholesteremic rabbit serum injected into normal rabbits produced lesions resembling atheroma. However, if the serum was centrifuged at 42,000 rpm for 5 hours and the upper and lower fractions injected separately, lesions were produced by the lower but not by the upper fraction, although both fractions contained appreciable amounts of cholesterol. These findings suggested that cholesterol associated with very low-density lipid particles may play little or no role in production of atherosclerosis. Kellner *et al.*(2) showed that intravenous administration of Triton inhibited development of atherosclerosis in cholesterol-fed rabbits. Forbes and Petterson(3) have reported the effects of centrifugation at 20,000 × g on lipid distribution of sera from rabbits receiving intravenous Triton WR 1339. A large proportion of total serum cholesterol, phospholipids, and neutral fat rose to the surface in contrast to minimal lipid migration in controls. Rabbits rendered markedly hypercholesteremic by cholesterol feeding and given Triton injections showed less cholesterol in the infranatant sera than was present in the infranate from the same animals before the Triton injections, in spite of the augmented hypercholesteremia produced by Triton. The protective effect of Triton in cholesterol-fed rabbits may have resulted from these changes observed in the serum lipid distributions.

Albrink, Man, and Peters(4) have reported that in severe hyperlipemia a considerable amount of the serum cholesterol rose to the surface during high-speed centrifugation. The present report is concerned with the effect of comparable centrifugation on lipid distribution of sera from a diversified group of patients, with particular emphasis on subjects with elevated serum neutral fat or other evidence of disturbed lipid metabolism. The clinical significance of the differences noted will not be discussed here.

Methods. 10 ml of clotted blood were obtained and the serum divided into 2 portions. One portion was centrifuged at 20,000 × g for 2 hours in a Sorval refrigerated centrifuge. A portion of the infranate was carefully removed with a sharp pointed pipet; lipids contained in this fraction are henceforth considered to be in the "soluble" form, consistent with the terminology established by Albrink, Man, and Peters(4). This fraction and the original whole serum were analyzed for cholesterol, neutral fat, and phospholipid phosphorus. A slight modification of a previously described procedure(5) requiring 0.2 ml of serum was used for cholesterol determinations, phospholipid phosphorus was determined by the method of Outhouse and Forbes(6), and neutral fat was determined directly using a modification(7) of the method of Van Handel and Zilversmit(8). In view of the work of many laboratories with similar centrifugation technics, the centrifugation conditions utilized in these studies can be assumed to result in flotation of the chylomicra and some beta-lipoproteins of very low density. Although a few of the determinations in this study were serial ones on the same subjects (particularly in the case of those with familial hyperlipemia), the majority were single determinations on separate subjects.

Results. The results presented in Table I show that among the miscellaneous group of subjects the per cent of cholesterol in the "soluble" form over a wide range of total cholesterol values remained fairly constant at a given neutral fat concentration. Thus, for example, when neutral fat was below 176 mg %, averages over various total cholesterol

TABLE I. Changes in "Soluble" Cholesterol as Related to Total Cholesterol and Neutral Fat Contents of Sera.

	Serum cholesterol ranges							
	<226 mg %		226–300 mg %		301–500 mg %		>500 mg %	
	Avg value (mg %)		Avg value (mg %)		Avg value (mg %)		Avg value (mg %)	
Neutral fat range (mg %)	Serum	Infranate	Serum	Infranate	Serum	Infranate	Serum	Infranate
Miscellaneous group								
<176	186 (37)	179 95*	259 (54)	251 96*	361 (42)	348 96*	532 (2)	519 97*
176– 300	193 (23)	185 95*	264 (23)	246 93*	353 (59)	326 92*	529 (7)	518 97*
301– 500	220 (8)	199 90*	263 (30)	237 90*	339 (44)	332 97*	608 (11)	547 89*
501– 800	203 (1)	160 78*	264 (9)	231 87*	388 (32)	335 86*	637 (16)	525 82*
801–1200	218 (1)	169 77*	249 (1)	107 42*	394 (16)	312 79*	673 (8)	505 73*
1200–2000	—	—	—	—	428 (6)	318 74*	637 (8)	408 64*
>2000	—	—	—	—	427 (3)	301 70*	864 (9)	349 40*
Familial hyperlipemia								
1200–2000	—	—	269 (5)	57 29*	337 (11)	49 14*	884 (5)	100 11*
>2000	—	—	282 (3)	41 14*	—	—	—	—

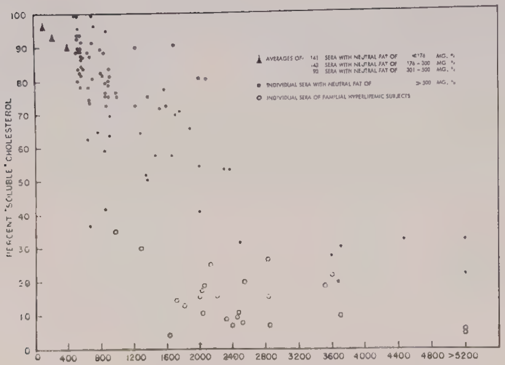
( ) = No. of determinations. * % total serum cholesterol.

ranges revealed that 95 to 97% of serum cholesterol was "soluble," even in sera with total cholesterols over 500 mg %. This relationship between "soluble" and total cholesterol was however subject to considerable individual variability and became less and less uniform as both neutral fat and total cholesterol concentrations increased. Sera from 3 subjects with familial hyperlipemia consistently showed exceptionally small amounts of "soluble" cholesterol.

As neutral fat concentrations increased, progressively more cholesterol rose to the surface with resultant steady decreases in per cent of total cholesterol in the "soluble" state. This inverse relationship between neutral fat and per cent "soluble" cholesterol is further illustrated in Fig. 1. The data suggested the possibility that familial hyperlipemics had somewhat lower amounts of "soluble" cholesterol at a given neutral fat concentration than subjects with other forms of hyperlipemia.

The relationship between total and "soluble" neutral fat is presented in Fig. 2. The general linearity illustrated by the vast majority of subjects indicated that "soluble" neutral fat increased proportionally as total neutral fat increased, at least up to a whole serum concentration of 2000 mg %. However, it was striking to observe the departure from this general linearity exhibited by subjects with familial hyperlipemia, even at whole serum concentrations well below 2000 mg % (2 subjects with acute pancreatitis showed cholesterol and neutral fat concentrations and distributions similar to that of the familial hyperlipemics; however, the lipid determinations approached normal with clinical improvement). Concerning those few hyperlipemic subjects from the "miscellaneous" group which fell among the familial hyperlipemic subjects in Fig. 1 and 2, the possibility exists that they also had the familial disorder without verification of the familial aspect, or that they had idiopathic hyperlipemia closely related to the familial type.

Phospholipid phosphorus changes were followed in over 200 sera (Table II). In general,



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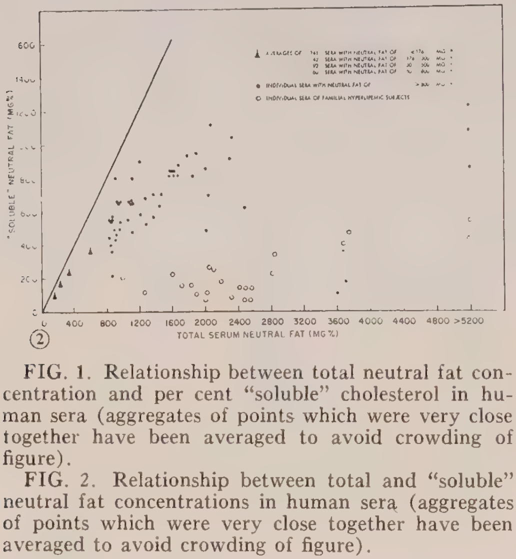

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FIG. 1. Relationship between total neutral fat concentration and per cent "soluble" cholesterol in human sera (aggregates of points which were very close together have been averaged to avoid crowding of figure).

FIG. 2. Relationship between total and "soluble" neutral fat concentrations in human sera (aggregates of points which were very close together have been averaged to avoid crowding of figure).

when neutral fat was below 176 mg %, very little, if any phospholipid or cholesterol rose to the surface. Although there was considerable individual variation, the average percentage change in cholesterol and phospholipid following centrifugation showed good agreement except in the case of the familial hyperlipemics where a much smaller percentage of cholesterol than of phospholipid was "soluble."

Discussion. The data presented do not permit evaluation of the physico-chemical association of the cholesterol undergoing flotation because the supernate was not further divided into chylomicra and very low-density lipoproteins. However, the work of a number of investigators suggested the possibility that some of the cholesterol undergoing flotation was associated as an integral part of chylomicra. Bragdon *et al.*(9,10) demonstrated that chylomicra, washed free of unassociated or loosely bound lipoproteins, contained small amounts of cholesterol, phospholipids, and protein, and that cholesterol content could be increased in experimental animals by cholesterol feeding. Lindgren *et al.*(11) suggested that chylomicra have a triglyceride "core" containing cholesterol and phospholipids surrounded by high density lipoproteins. The possibility of cholesterol-lipoprotein comlexes within chylomicra was suggested by the work of Rodbell and Fredrickson(12) demonstrating the association of alpha-lipoproteins with chylomicra and the work of Korn(13) showing that alpha-lipoproteins were required for lipoprotein lipase hydrolysis of a coconut oil emulsion.

The difference in flotation of cholesterol and neutral fat between the miscellaneous group and familial hyperlipemics with comparable neutral fat concentrations may be due to differences in the protein component of some of the lipoprotein complexes. Rodbell(14) found that the protein component of lipoproteins in the range of Sf 100-400 from patients with idiopathic hyperlipemia was characterized by peptides with N-terminal aspartic acid, serine and threonine. The N-terminal residue of this class in normal subjects was apparently

TABLE II. Changes in "Soluble" Cholesterol and Phospholipids as Related to Neutral Fat Contents of Sera.

Neutral fat range (mg %)	No. of determinations	Cholesterol			Phospholipid phosphorus		
		Serum (mg %)	Infranate (mg %)	% "soluble"	Serum (mg %)	Infranate (mg %)	% "soluble"
<176	85	265 ± 5.8*	256 ± 6.1*	96	8.5 ± .18*	8.2 ±.14*	96
176– 300	53	318 ± 16	303 ± 13	95	9.3 ± .28	9.0 ±.32	96
301– 500	37	354 ± 13	327 ± 13	92	11.3 ± .33	10.4 ±.31	92
501–1000	19	434 ± 30	346 ± 29	79	13.6 ± .78	11.1 ±.79	81
>1000	33†	458 ± 41	94 ± 16	20	13.5 ±1.0	6.4 ±.49	49

* Stand. error of mean.

† Familial hyperlipemic subjects.

glutamic acid(15). In idiopathic hyperlipemics, Furman(16) has shown that lipoproteins with the characteristics of alpha$_1$-lipoproteins can be dissociated by sonic vibration from the low-density beta-lipoprotein fraction separated at D 1.006.

Since there is evidence to suggest that chylomicron cholesterol plays little or no role in atherogenesis(17), further studies are in progress to determine if the presence of clinical atherosclerosis correlates better with "soluble" than with total serum cholesterol.

Summary. The effects of centrifugation at 20,000 × g for 2 hours on lipid distribution of various human sera have been studied. In general, when neutral fat was below 176 mg %, very little, if any, cholesterol or phospholipids rose to the surface. When neutral fat content was markedly elevated, as much as 90% of both cholesterol and neutral fat and 50% of phospholipids underwent flotation. However, subjects with similar degrees of marked hyperlipemia appeared to separate into 2 major groups represented by moderate and marked degrees of flotation of neutral fat, the latter group being composed primarily of subjects with familial hyperlipemia. An inverse correlation was found between whole serum neutral fat content and percentage of total cholesterol remaining in the subnatant serum. Variations from these general patterns and possible clinical interpretations are being studied.

We gratefully acknowledge a grant from the Richmond Area Heart Assn. in support of our work on lipid metabolism. Our thanks are due Dr. William Burton and Dr. Morton Bender as well as many practicing physicians in Richmond for collecting blood samples used in this study.

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1. Bragdon, J. H., *Circulation,* 1951, v4, 466.

2. Kellner, A., Cornell, J. W., Ladd, A. T., *J. Exp. Med.,* 1951, v93, 385.

3. Forbes, J. C., Petterson, O. M., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 368.

4. Albrink, M. J., Man, E. B., Peters, J. P., *J. Clin. Invest.,* 1955, v34, 147.

5. Forbes, J. C., *J. Lab. Clin. Med.,* 1931, v16, 520.

6. Outhouse, E. L., Forbes, J. C., *ibid.,* 1940, v25, 1157.

7. Forbes, J. C., Forbes, A. L., Petterson, O. M., PROC. SOC. EXP. BIOL. AND MED., 1960, v105, 628.

8. Van Handel, E., Zilversmit, D. B., *J. Lab. Clin. Med.,* 1957, v50, 152.

9. Bragdon, J. H., Havel, R. J., Boyle, E., *ibid.,* 1956, v48, 36.

10. Bragdon, J. H., *Ann. N. Y. Acad. Sci.,* 1959, v72, 845.

11. Lindgren, F. T., Nichols, A. V., Hayes, T. L., Freeman, N. K., Gofman, J. W., *ibid.,* 1959, v72, 826.

12. Rodbell, M., Fredrickson, D. S., *Fed. Proc.,* 1958, v17, 298.

13. Korn, E. D., *J. Biol. Chem.,* 1955, v215, 15.

14. Rodbell, M., *Science,* 1958, v127, 701.

15. Avigan, J., Redfield, R., Steinberg, D., *Biochem. et Biophys. Acta.,* 1956, v20, 557.

16. Furman, R. H., quoted by Olson, R. E., Vester, J. W., *Physiol. Rev.,* 1960, v40, 677.

17. Olson, R. E., Vester, J. W., *ibid.,* 1960, v40, 677.

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On the Hemolytic Activity of Mouse Complement.* (26586)

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As judged by the usual hemolytic tests, mouse serum appears to lack complement (C′). By use of a reaction system containing a very low concentration of sensitized cells, McGhee(1) demonstrated one unit of hemolytic C′ activity per ml in 7 of 12 mouse sera, and 2 units per ml in the serum of a single animal, but even with his sensitive test system, 4 out of 12 sera had no hemolytic activity. According to Ritz(2), the first component, C′1, is present since EA treated with mouse serum can be hemolyzed by subsequent incubation with guinea pig endpiece. Brown

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(3) claimed that C′3 and C′4 are also present, and that only C′2 is missing. Rice and Crowson(4) did not find C′2, C′3 or C′4 in tests with R2, R3, or R4 made from guinea pig serum; Ritz's demonstration of C′1 was confirmed. The finding by Marcus *et al.*(5) that mouse serum lacks bactericidal activity against certain Gram-negative bacteria was confirmed by Muschel and Muto(6), who showed, in addition, by bactericidal as well as hemolytic tests that C′2 and C′3 appear to be absent. Thus, there is agreement as to presence of C′1 and absence of C′2, but with regard to C′3 and C′4 results are contradictory.

The inadequacy of R1, R2, R3 and R4 as reagents for detection and measurement of the C′ components has been pointed out(7) and, furthermore, attention has been drawn to the presence of inhibitory factors in fresh blood serum which may mask the cytotoxic activity of the C′ system unless their interference is circumvented by appropriate design of the test system. Through detailed studies of the reaction mechanism of guinea pig as well as human C′, improved analytical methods for detection and measurement of the C′ components have been developed by use of the intermediates as substrates(7). We have initiated a reexamination of mouse C′ by these new technical procedures, because mice are used widely in studies of the immunologic aspects of tissue transplantation, tumor rejection and resistance to microbial infection. The early results of this reinvestigation, presented herewith, indicate that all of the C′ factors are present in mouse serum.

Materials and methods. Sheep erythrocytes (E), rabbit antibody (A) to boiled sheep erythrocyte stromata, and the diluent, veronal-buffered saline containing 0.00015 *M* Ca^{++} and 0.0005 *M* Mg^{++}, were prepared as described in(7). Technics for preparation of the intermediate EAC′1,4 and for separation of g.p. C′2 in a state of functional purity are given in(7,8). EAC′4(9) was made by treatment of 1.0 ml of EAC′1,4 (10^9 E/ml) with 0.11 ml of 0.1 *M* EDTA (ethylenediaminetetraacetate, pH 7.7) at 37°C for about 5 minutes. The cells were washed twice with cold diluent followed by suspension in sufficient diluent to make a cell concentration of 1.5×10^8 per ml. They may be stored at 0°C for about one week without loss of $SA_2C'4$ sites, but it is necessary to wash the cells and adjust their concentration just prior to use for detection and measurement of C′1. The theoretical principles and technical procedures for titration of C′ components on molecular basis by use of the intermediates as substrates are described in(7,8,10). Reaction temperatures were maintained by use of water baths controlled within ± 0.02°C.

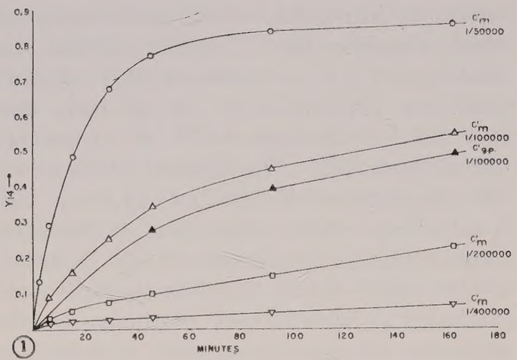
Collection of mouse serum. The jugular veins of adult white CFW mice were incised and the animals exsanguinated into a large Petri dish resting on crushed ice. Blood from about 45 mice was pooled. Clotting was rapid, and immediately after bleeding the last animal of the group, the blood was transferred to plastic tubes and centrifuged at 8,000 RCF for 30 minutes at 2-4°C. The serum was removed by pipette, centrifuged again for 15 minutes at 2-4°C at 1,000 RCF, distributed in test tubes, 0.5 ml per tube, and stored at −50°C.

Serum obtained in this fashion contained 2 to 3 times more C′1 than mouse serum from pooled blood allowed to clot at room temperature. The loss of C′1 on clotting at room temperature is probably due to fixation by isoantibody-isoantigen interaction. This difficulty could be avoided by pooling *after* careful separation of each individual serum from cells, but this is technically cumbersome and therefore we adopted the practice of immediate chilling and prompt centrifugation. Clotting appeared to be complete since the serum collected as outlined above showed no clot formation on incubation at 37°C.

Experimental and results. C′1. Becker (9) showed that EAC′4 does not react with C′2 to form EAC′1,4,2, unless treated with C′1. Thus, EAC′4 can be used for measurement of C′1. It should be noted that the EAC′4 cells used in the present experiments carry SA_2 as well as $SA_2C'4$ sites. Hence, on reaction with C′1, both $SA_2C'1$ and $SA_2C'1,4$ sites may be formed, but only the latter will react with C′2 to yield $SA_2C'1,4,2$. This results in waste of some C′1, the extent of loss depending on the ratio of SA_2 to $SA_2C'4$. Analyses for C′1 made with different preparations of EAC′4 will be comparable only if this ratio is kept constant.

Experiment MC020161. Ten ml portions of EAC′4 (1.5×10^8 E/ml) were mixed with equal volumes of whole mouse serum diluted 1/50,000, 1/100,000, 1/200,000, 1/400,000, and the mixtures were incubated at 37.0°C with gentle mechanical agitation. One ml samples, removed at suitable time intervals, were delivered into and mixed promptly with 5.0 ml ice-cold diluent to stop the reaction. The diluted samples were centrifuged immediately at 4°C, the clear supernatant fluids were removed as completely as possible by decantation and thorough drainage, and the cells were suspended in 1.0 ml of purified g.p. C′2 diluted to supply about 200 effective molecules of C′2 per cell. The reaction mixtures were incubated at 30.0°C for about 30 minutes, which suffices for complete conversion of $SA_2C'1,4$ to $SA_2C'1,4,2$, then 1.5 ml of a 1/37.5 dilution of g.p. C′ in 0.005 *M* EDTA buffer (C′-EDTA) were added. After 90 minutes' incubation at 37.0°C for conversion of $SA_2C'1,4,2$ to S*, followed by lysis of all cells with at least one S*, 5.0 ml of 0.15 *M* NaCl were added with mixing, the tubes were centrifuged to sediment the cells and hemoglobin concentration of the supernatant fluids was measured photometrically at 412 mμ to determine degree of hemolysis. Results were corrected by subtraction of the appropriate controls which included: EAC′4 + diluent, EAC′4 + 1/50,000 mouse C′, EAC′4 + 1/50,000 mouse C′ + C′2, EAC′4 + 1/50,000 mouse C′ + C′-EDTA. The control flask containing EAC′4 + diluent showed less than 1% lysis at the end of experiment, and none of the other controls exceeded this value. From the results shown in Fig. 1 it is evident that mouse serum is a potent source of C′1. The C′1 activity is quantitatively comparable to that of guinea pig serum which has been estimated to contain 9×10^{12} effective molecules of C′1 per ml(10). The data in Fig. 1 show the kinetics of reaction in terms of $y_{1,4} \rightarrow$ which denotes the fraction of cells in the state EAC′1,4 or further in the sequence of successive reactions which comprise the C′ system. Thus, the symbol EAC′1,4 → refers to cells with at least one $SA_2C'1,4$ site, or at least one $SA_2C'1,4,2$, or at least one S*, or a combination of these. *C′2. (Experiment MC021361)* Six ml portions of EAC′1,4 (1.5×10^8 E/ml) were mixed with equal volumes of whole mouse serum diluted 1/1,000, 1/2,000, 1/4,000 and 1/8,000. Reaction mixtures were incubated at 30.0°C with continuous gentle agitation. At appropriate time intervals 1.0 ml samples were removed, mixed with 1.5 ml of g.p. C′-EDTA and incubated for 90 minutes at 37°C, followed by measurement of degree of lysis. Results were corrected by subtraction of appropriate controls which included: EAC′1,4 + diluent, EAC′1,4 + mouse C′ 1/1,000, EAC′1,4 + C′-EDTA. None of the controls had more than 1% lysis. The corrected data are shown in Fig. 2 as a plot of $y_{1,4,2} \rightarrow$, the proportion of cells in the state EAC′1,4,2 →, as a function of time of incubation at 30.0°C following addition of the mouse serum dilutions to EAC′1,4. For comparison, 2 similar experiments with guinea pig serum are included, one showing reaction of EAC′1,4 with a 1/2,000 dilution of purified C′2, the other depicting reaction of EAC′1,4 with a 1/12,000 dilution of whole g.p. C′.

These results indicate, of course, that mouse serum contains C′2, though less than guinea pig serum. The precise quantity cannot be estimated because of several complications, notably the difference in decay behavior between $EAC'1^{g.p.}4^{g.p.}2^{g.p.}$ and $EAC'1^{g.p.}4^{g.p.}2^{m}$. Fig. 2 shows that the former decays in a typical fade-away fashion, *i.e.*, closely approximating first-order kinetics with respect to loss of $SA_2C'1,4,2$ sites. The latter, on the other hand, shows no decay at all at the 1/8,000 mouse C′ dilution and only partial decay with the lower dilutions. These matters will be considered in the discussion. *C′4. (Experiment MC020261)* This component was demonstrated in terms of EAC′1,4,2 → formation on treatment of EA with mouse C′ as follows: Flask A was charged with 6.0 ml EA (1.5×10^8 cells/ml) and 6.0 ml of mouse C′ 1/750; Flask B received 15.0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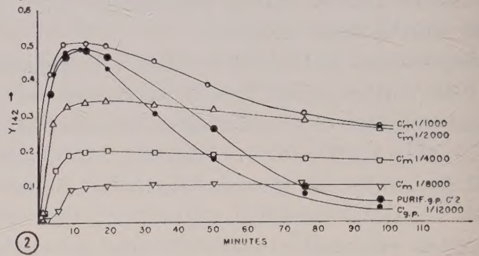
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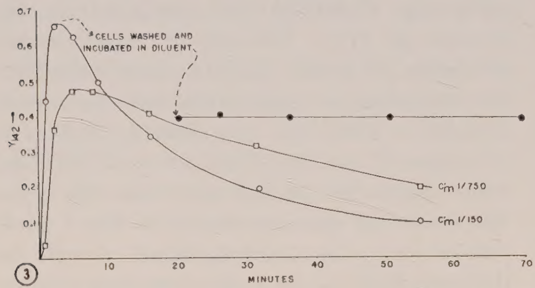
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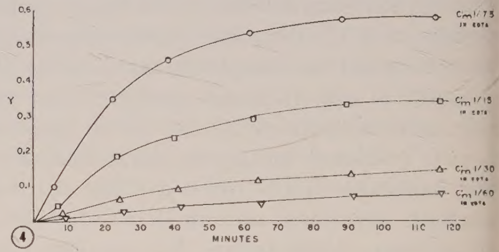
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FIG. 1. Kinetics of reaction of g.p. EAC′4 with whole mouse or g.p. serum at 37.0°C.

FIG. 2. Kinetics of reaction of g.p. EAC′1,4 with mouse or g.p. serum at 30.0°C. The purified g.p. C′2 furnished 1.35 effective molecules of C′2/cell.

FIG. 3. Kinetics of reaction of EA with whole mouse serum at 30.0°C. Solid circles represent a twice washed sample from a mixture of EA and C'_m 1/150 which was incubated at 37.0°C and sampled into g.p. C′-EDTA.

FIG. 4. Kinetics of reaction of guinea pig EAC′1,4,2 with mouse C′-EDTA at 37.0°C.

ml of EA (1.5×10^8 cells/ml) and 15.0 ml of mouse C′ 1/150 (a larger quantity of reaction mixture was used in Flask B in order to furnish sufficient material for a subsequent step in the experiment, as described below). Reaction mixtures were incubated at 30.0°C with gentle mechanical rocking. Samples of 1.0 ml were collected and mixed promptly with 1.5 ml of g.p. C′-EDTA, followed by incubation at 37.0°C for 90 minutes and photometric determination of degree of lysis in the usual manner. Controls, set up and sampled as in the other experiments, showed less than 1% lysyis at beginning and end of the experiment.

From preliminary experiments it was suspected that mouse serum contains a factor which destroys $SA_2C'1,4,2$ sites. To demonstrate this effect, 10.0 ml of reaction mixture were removed from Flask B at about 4 minutes, the cells were sedimented by centrifugation, washed twice with diluent at room temperature and suspended in 10.0 ml of diluent. This cell suspension was incubated at 37.0°C with agitation and 1.0 ml samples were mixed with 1.5 ml of g.p. C′-EDTA followed by incubation at 37.0°C for 90 minutes and photometric determination of extent of lysis.

The results, shown in Fig. 3, as a plot of $y_{1,4,2} \rightarrow$ *vs.* time, indicate that mouse serum contains C′4, as well as C′1 and C′2. It should be noted that the portion of cells removed from the reaction mixture at 4 minutes did not decay on incubation at 37°C. The interpretation of this effect will be taken up in the discussion. *C′3. (Experiment MC021561).* Initial attempts to demonstrate C′3 were made by incubating washed $EAC'1^m4^m2^m \rightarrow$ (*i.e.*, non-decaying) with a 1/37.5 dilution of mouse serum in 0.01 *M* EDTA-diluent, but these were unsuccessful. Appropriate tests after 9 minutes' incubation at 37.0°C revealed that the cells were not EAC′1,4, nor EAC′1,4,2 nor EAC′4, indicating destruction of the reactive sites by mouse C′-EDTA.

In view of this, subsequent experiments were performed with $\mathrm{EAC'1^{g.p.}4^{g.p.}2^{g.p.}}$ as substrate. Four ml of $\mathrm{EAC'1^{g.p.}4^{g.p.}}$ (10^9 cells/ml, $t_{max} = 13$ min) were treated with 4.0 ml purified g.p. C′2 (furnishing about 300 C′2 per cell) for 13 minutes at 30°C. EAC′1,4,2 prepared in this manner carry approximately 40 to 50 $\mathrm{SA_2C'1,4,2}$ sites per cell. After washing in the cold, the cells were suspended in 0.01 *M* EDTA-diluent to a concentration of 1.5×10^8 cells/ml. Seven ml aliquots of this suspension were mixed with 10.5 ml portions of mouse serum diluted 1/7.5, 1/15, 1/30, 1/60 in 0.01 *M* EDTA-diluent, and incubated with gentle rocking at 37.0°C. Samples of 2.5 ml were pipetted into 5.0 ml portions of ice-cold 0.15 *M* NaCl, followed immediately by centrifugation in the cold and subsequent determination of degree of lysis. As a control, $\mathrm{EAC'1^{g.p.}4^{g.p.}}$ were incubated with mouse C′-EDTA, diluted 1/7.5 and sampled in the same manner as the experiment proper. The extent of lysis in these control samples was exactly equal to degree of spontaneous cell lysis, which was less than 1%. The results plotted in Fig. 4, in terms of degree of lysis as a function of time of incubation, indicate presence of C′3 activity in mouse serum, though of much lower potency than that exhibited by g.p. serum. It should be noted that C′3 is defined as a group of several factors which lyse EAC′1,4,2; 3 distinct factors in guinea pig serum have been demonstrated by Rapp(11) and Taylor and Leon have made similar observations with human serum(12). It appears likely that mouse C′3 also comprises more than one factor, since a plot of the extent of lysis of EAC′1,4,2 at 120 minutes (*cf.* Fig. 4) as a function of concentration of mouse C′-EDTA yielded a sigmoidal curve.

Discussion. The present experiments, though crude and lacking quantitative refinement, demonstrate unequivocally that mouse serum contains all of the recognized factors of the C′ system, despite the fact, observed by previous investigators, that over-all hemolytic activity of C′ is practically nil. It is not difficult to find reasons for the poor over-all C′ activity. First, and possibly most important, the C′2 and C′3 activities of mouse serum appear to be significantly lower than in g.p. serum. In addition, C′4 activity is lower, but it is not known whether this plays a significant role. Furthermore, mouse serum contains a factor which destroys $\mathrm{SA_2C'1,4,2}$.

The inability of mouse C′-EDTA to lyse $\mathrm{EAC'1^{m}4^{m}2^{m}}$, like the lack of over-all hemolytic activity of mouse C′, probably can be attributed to low multiplicity of $\mathrm{SA_2C'1,4,2}$, low concentration of one or more of the C′3 components and destruction of $\mathrm{SA_2C'1,4,2}$ by a factor in mouse serum. As a result, the probability of S* formation is low. On the other hand, mouse C′-EDTA was able to lyse $\mathrm{EAC'1^{g.p.}4^{g.p.}2^{g.p.}}$, presumably because this substrate had a multiplicity of about 40 to 50 $\mathrm{SA_2C'1,4,2}$.

These statements are tentative, subject to revision as more information becomes available on the concentration of each of the C′ factors in mouse serum, on optimal conditions with respect to temperature, concentration of reagents, time of incubation, ionic strength, concentrations of Ca^{++} and Mg^{++}, etc., applicable to each of the mouse C′ factors. Furthermore, there is no information on the efficiency of substitution of mouse C′ components for those of g.p. serum.

One of the most interesting and significant observations in these experiments is that $\mathrm{EAC'1^{m}4^{m}2^{m}} \rightarrow$ does not decay on incubation at 37°C. As stated above, the arrow in this symbol indicates that the cells are in the state EAC′1,4,2 or beyond, *i.e.*, somewhere in the C′3 stage. The reason for introducing this new symbol derives from the definition of C′3, namely, a group of factors which convert EAC′1,4,2 to E*. It is obvious that the converse is not true, *i.e.*, cells which are converted to E* by treatment with the C′3 factors can be in the state EAC′1,4,2, or beyond. Thus, the new symbol EAC′1,4,2 →, denotes cells which are lysable by C′-EDTA.

Müller-Eberhard(13) showed that a glycoprotein from fresh human serum, designated β_{1C}, stabilizes $\mathrm{EAC'1^{g.p.}4^{g.p.}2^{g.p.}}$, and a likely interpretation of this observation is the assumption that β_{1C} is part of the group of C′3 factors and that its reaction with EAC′1, 4,2 produces a stable successor. The present observation that EAC$'1^{m}4^{m}2^{m}$, if washed to remove the destructive mouse serum factor, does not decay on incubation at 37°C, might be interpreted along the same lines, *i.e.*, that this intermediate is not EAC'1,4,2 but represents a state analogous to that formed on interaction of EAC'1,4,2 with Müller-Eberhard's β_{1C}. Admittedly, there are other possible interpretations, including the proposition that EAC$'1^{m}4^{m}2^{m}$ is inherently stable in contrast to EAC$'1^{g.p.}4^{g.p.}2^{g.p.}$, but on the basis of present information the hypothesis of stabilization by one of the mouse C'3 factors appears more reasonable.

Summary. By use of appropriate intermediates of the complement system, mouse serum has been shown to contain C'1, C'4, C'2 and the C'3 complex.

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1. McGhee, R. B., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 419.

2. Ritz, H., *Ztschr. f. Immunitaetsf.*, Orig., 1911, v9, 321.

3. Brown, G. C., *J. Immunol.*, 1943, v46, 319.

4. Rice, C. E., Crowson, C. N., *J. Immunol.*, 1950, v65, 201.

5. Marcus, S., Esplin, P. W., Donaldson, D. M., *Science*, 1954, v119, 877.

6. Muschel, L. H., Muto, T., *Science*, 1956, v123, 62.

7. Kabat, E. A., Mayer, M. M., *Experimental Immunochemistry*, C. C Thomas, Springfield, Illinois, 1961.

8. Borsos, T., Rapp, H. J., Mayer, M. M., I - III, *J. Immunol.*, 1961, v87.

9. Becker, E. L., *J. Immunol.*, 1960, v84, 299.

10. Hoffman, L. G., Purification of the First and Fourth Components of Guinea Pig Complement and Studies on their Mechanism of Action, Dissertation, The Johns Hopkins University School of Hygiene and Public Health, 1960.

11. Rapp, H. J., *Science*, 1958, v127, 234, and unpublished evidence on three C'3 factors.

12. Taylor, A. B., Leon, M. A., *Fed. Proc.*, 1961, v20, 19.

13. Müller-Eberhard, H. J., Nilsson, U., *J. Exp. Med.*, 1960, v111, 217.

Received March 28, 1961. P.S.E.B.M., 1961, v107.